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STUDIES ON THE EXCYSTMENT OF COLPODA CUCULLUS

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I. INTRODUCTION AND LITERATURE

THE undried, permanent cysts of *Colpoda cucullus* may be quantitatively changed back into the free-swimming form under suitable conditions. This ciliate does not excyst from its permanent cysts spontaneously, but must be stimulated to excyst by some very definite change in the environment. The first problem is that of determining what kinds and degrees of stimuli will induce the excystment response. In this paper a technique for approaching this problem is described, as are also certain results, which, though very incomplete, are nevertheless sufficiently striking and suggestive to serve as a starting-point for further investigations into the relation between excystment and the chemical environment.

The study of the environmental changes which induce excystment in Protozoa is relatively new, and little is known with certainty. Koffmann (1924) claims to have shown that a sudden drop of pH from 8.4 to 6.0 is sufficient to cause complete excystment in *Colpoda steinii* and *C. cucullus*, but his evidence is very faulty and not at all conclusive. Garnjobst (1928) made the observation that the hypotrich *Euplotes taylori*, after having been made to encyst by evaporation of the culture medium, could be induced to excyst by again diluting the medium with distilled water. Darby (1929) supports Koffmann's opinion. His experiments on *Stylonychia pustulata* indicate that excystment in this organism occurs only in the pH region of approximately 6.4–8.5. The most important contribution in this field is the work of Weyer (1930) on *Gastrostyla*. He concludes, as a result of an extensive series of experiments, that the organic constituents of the medium are solely responsible for inducing excystment in *Gastrostyla;* and he attempts to identify the active organic constituents with the end-products of protein decomposition by bacteria. It will be seen in the following pages that the results reported here are in essential agreement with the conclusions of Weyer as to the importance of organic materials for inducing excystment. But the specific identity of the active substance or substances remains to be discovered.

TECHNIQUE

In order to study the conditions of excystment, it is necessary to obtain large numbers of cysts which are essentially similar in respect to their response to various excystment stimuli, and also to keep these in a known and reproducible environment previous to the experiments. An attempt to produce such a more or less standard cyst-preparation was made by using the following method:

Finger bowls containing 10–20 cc. of a dilute hay infusion are inoculated with *Colpoda* from a single clone and are allowed to stand until a great number of organisms has developed and then completely encysted in the bottoms and walls. Such adhering permanent cysts (Barker and Taylor, 1931) are washed largely free of adhering bacteria and débris by allowing tap water to run gently over the dish. They are then just covered with fresh dilute hay infusion and left until they have mostly excysted. This requires from 2 to 4 hours. After excystment the rather dense suspension of free-swimming *Colpoda* is centrifuged at low speeds, the supernatant fluid poured off, and the remainder diluted with fresh balanced medium and centrifuged again. This washing process is repeated three or four times until the hay infusion has been quite completely replaced by a purely inorganic medium which is buffered weakly to a pH of 7. The final suspension is made up to such a volume (5 cc.) that there are approximately 3,000–5,000 organisms per cubic centimeter of fluid. One-fourth cubic centimeter of such a suspension is placed in each of a number of ground-glass depression slides, and these are stacked in a moist chamber until such time as the *Colpoda* encyst. Two days are generally sufficient for complete encystment under these conditions, and this time may be used as a standard time for preparing cysts for excystment experiments.

Such a method is easily carried out and yields a material always essentially similar in respect to concentration of organisms, composition of medium, rate of excystment, and, indeed, most of those factors of immediate environment and previous history which might conceivably influence excystment. Despite this, some variation in the sensitiveness to excystment stimuli is observed in different lots of cysts.

After having obtained such a standard cyst-preparation, great care must still be taken with the method for testing the excystment-inducing activity of various materials. This will be made clear from the following discussion:

If the medium is removed from the standard cyst-preparation and replaced by the same volume of a dilute hay infusion, all or nearly all of the organisms excyst within 1–2½ hours.

The replacement of the cyst medium by hay infusion, simple though it seems, involves several possibilities of stimulation which might vitiate the interpretation of the experiment. The old medium must be removed from above the cysts; this requires a certain amount of mechanical agitation. Also, the cysts are unavoidably left exposed to the air for a short time covered only by a thin film of water. The question presents itself as to whether we might not just as well replace the old medium after these manipulations with the same result as is obtained with the hay infusion. Experiments carried out to test the stimulation inherent in the manipulation indicate that: (1) mechanical disturbance and aëration of the cyst medium produced by blowing bubbles through it does, in fact, act in a small minority of instances as a weak excystment stimulus; (2) brief exposure (1–2 minutes) of cysts to air when covered by only a surface film of water, followed by replacement of the old medium, stimulates the more responsive cysts to excystment; (3) replacement of the encystment medium by fresh balanced medium—the same in which the organisms originally encysted—may or may not induce excystment. It occasionally does do so. Evidently, then, the precise technique of these experiments is of considerable importance.

In order to test the stimulating effect of materials in solution, a technique less open to complications has been used. Instead of removing the old medium from the cysts, the procedure is simply to add 1 drop ($\frac{1}{16}$ cc.) of the solution to be tested to the cysts as they stand. Even this method requires the simultaneous running of three control experiments. One control dish of cysts is left untouched, to make sure that spontaneous excystment or the excystment due to temperature changes, etc.—unavoidable stimuli inherent in the manipulation—does not occur. A drop of a 1 per cent hay infusion or yeast extract, known to induce excystment, is added to a second control to see if the cysts actually are viable and normally responsive. To the third control is added a solution identical in amount and similar in all such factors as osmotic pressure, salt balance, pH, etc., to the solution to be tested and lacking only the one material whose stimulating effects are under observation. This last type of control experiment is absolutely essential, since it has been at times observed that the addition of a single drop of balanced medium to the 4 drops ($\frac{1}{4}$ cc.) of medium containing cysts caused from a slight to a very considerable excystment response.

Using all of these controls, the method has then been to add a single drop ($\frac{1}{16}$ cc.) of a solution of known strength of the material whose excystment-inducing activity is being studied to the standard cyst-preparation and to observe at various time-intervals thereafter approximately what proportion of the *Colpoda* actually have excysted. Importance has been attached to the actual percentage of organisms which excyst within a reasonable time, rather than to the absolute rate of excystment. This may be justified by the fact that the rate of excystment appears to be quite independent of the strength of the excystment stimulus.

EXPERIMENTAL RESULTS

In order to simplify and abbreviate the presentation of our experimental results, only one typical experiment will be recorded in full. The bulk of the experimental data will be summarized in a more condensed form.

In this typical experiment (Table I) cysts were prepared in twenty-two depression slides according to the above-described method. Each slide contained $\frac{1}{4}$ cc. of liquid and approximately 1,000 organisms. After 2 days, practically 100 per cent of the *Colpoda* had encysted. At 11:00 A.M. of the second day, approximately $\frac{1}{16}$ cc. (1 drop) of the solution indicated was added to the respective cyst-preparations. Estimations were made at intervals with the aid of a low-magnification dissecting microscope of the percentage of the organisms which had excysted. One such set of estimations, made at 3:30 P.M., i.e., after $4\frac{1}{2}$ hours, will suffice to show the effect of the added materials. The maximum inaccuracy of the estimations is probably never more than ± 10 per cent; and in the regions of very high and very low percentages, it is undoubtedly much less, possibly ± 2 per cent.

TABLE I

THE DIFFERENT REACTIONS OF *Colpoda* CYSTS TO VARIOUS MATERIALS IN SOLUTION

Slide No.	11:00 A.M. Solution Added (1/16 cc.)	3:30 P.M. Percentage of Excystment
1	Balanced salt medium pH 7	0
2	Dilute alfalfa hay infusion	100
3	Dilute alfalfa hay infusion	100
4	Dilute (1/10 per cent by weight) yeast-autolysate	95
5	Dilute (1/10 per cent by weight) yeast-autolysate	90
6	Dilute (1/10 per cent by weight) malt extract	70
7	Dilute (1/10 per cent by weight) malt extract	60
8	1/10 per cent peptone solution	70
9	1/10 per cent peptone solution	75
10	Saturated methyl salicylate solution	0
11	Saturated methyl salicylate solution	0
12	1/10 per cent saponin solution	0
13	1/10 per cent saponin solution	0
14	M/30 sodium phosphate pH 6.4	0
15	M/30 sodium phosphate pH 6.4	0
16	M/30 sodium phosphate pH 8.0	0
17	M/30 sodium phosphate pH 8.0	0
18	M/100 sodium laurate pH 7.5	0
19	M/100 sodium laurate pH 7.5	0
20	1 per cent amyl alcohol	0
21	1 per cent amyl alcohol	0

It is to be noted that the indicated solutions are made up in conformity with the principles of the preceding section, in a balanced salt medium of pH 7 and not in water merely. Slide No. 1 may be taken as a control on spontaneous excystment due to undefined cases and excystment due to dilution of the cyst-medium with balanced salt solution. Any of the slides Nos. 2–8, inclusive, are controls on viability and normal responsiveness to excystment stimuli.

It will be observed that certain materials may very markedly induce excystment while others are entirely inactive. In Table II are listed all of the materials tested which, when added in not too great dilution ($\frac{1}{10}$–1 per cent solutions), invariably induce excystment.

TABLE II

MATERIALS WHICH INVARIABLY INDUCE EXCYSTMENT

Timothy hay infusion
Alfalfa hay infusion
95 per cent alcohol-soluble fraction of hay infusion
Wheat (seed) infusion
Yeast extract
98 per cent alcohol-soluble and -insoluble fractions of dried yeast-autolysate
Ether-soluble fraction of yeast-autolysate
$CHCl_3$-soluble fraction of yeast-autolysate
Acid-acetone-soluble fraction of dried yeast-extract
Malt extract
Peptone solution
Beef extract
Banana extract

By far the greater number of materials tested are completely inactive. Table III lists the inactive, inorganic materials; Table IV, the organic materials.

TABLE III

SOLUTIONS WHICH DO NOT INDUCE EXCYSTMENT

M/30 sodium phosphate pH 6.4, pH 6.0
M/30 sodium phosphate pH 8.0, pH 8.4
M/100 NaCl pH 7.0
M/100 $CaCl_2$ pH 6.2
M/100 KCl pH 7.0
M/100 $NaHCO_3$
M/100 Na_2S
M/100 Na borate
M/100 Na iodate
Yeast salts (incinerated yeast-extract) approx. M/50

Only one substance gave uncertain results in these experiments. A saturated water solution of methyl salicylate sometimes caused marked excystment and at other times was quite inactive, as in the detailed experiment given above. So far, the cause of this variability of action has not been determined.

TABLE IV

ORGANIC MATERIALS WHICH DO NOT INDUCE EXCYSTMENT (APPROXIMATELY M/100 SOLUTIONS MADE UP IN BALANCED SALT MEDIUM UNLESS OTHERWISE SPECIFIED)

Lipoid-soluble and surface-active materials:

Caprylic alcohol
Saponin
Na laurate
Toluene (sat. sol.)
Ether
Amyl alcohol
Ethyl alcohol
Ethyl butyrate (sat. sol.)
Butyric acid
Neutral red

Foods:

Glucose (M/32 – M/1,000)
Ammonium glycero phosphate
Ammonium glycero lactate
Lactose
Ca lactate
Galactose
Levulose
Na pyruvate

Waste products of nitrogen metabolism:

Urea
Uric acid

Materials affecting oxidation-reduction potentials ($\frac{1}{10}$ *per cent solutions*):

Glutathione
Methylene blue
(oxidized and reduced forms)
Phenol indophenol (oxid.)
o-Chloro phenol indophenol (oxid.)
o-Cresol indophenol (oxid.)
Napthol 2-sulfonate indophenol
(oxid.)

Miscellaneous:

Gum guaiac
Na salicylate
Caffeine (very dilute solution)
Na formate

Besides these purely qualitative experiments, a group of semi-quantitative experiments were carried out in an attempt to fractionate dried yeast-extract in such a way as to obtain a preparation of greater excystment inducing activity.

First a series of solutions of yeast-extract ranging from 1 per cent to $\frac{1}{100}$ per cent by weight were tested for activity. It was found that solutions more concentrated than approximately $\frac{1}{50}$ per cent would induce excystment, while more dilute solutions would not do so.

These fractions of the dried yeast-extract were prepared in the following manner:

Fifteen grams of yeast-extract, dried at 100°–110° C. and powdered in a mortar, were extracted with 98 per cent alcohol in a Soxhlet extraction apparatus until the extract was substantially complete. One hundred and fifty cubic centimeters of alcohol were used. The alcohol solution was cooled to 20° C. and filtered to separate off the material insoluble in cold alcohol. This fraction, after being washed with cold alcohol, was dried at 110° C. and found to weigh 1.91 gm. Water solutions (1) were prepared in concentrations of $1-\frac{1}{100}$ per cent. The alcohol filtrate described above was evaporated to remove the alcohol. Six grams of solid remained. Solutions (2) of this were prepared, as were also solutions of the material remaining in the Soxhlet extraction (3) thimble.

The activity of these three fractions was compared with that of the original dried yeast material. No appreciable increase or decrease of activity could be demonstrated for any of these. All solutions of the same concentration showed approximately the same activity.

DISCUSSION

The striking result of these experiments is that only those materials which may be extracted from plant or animal tissues are active in inducing the excystment of *Colpoda cucullus* from its resting cysts. All pure chemicals, whether inorganic or organic (with the possible exception of methyl salicylate), appear to be completely inactive.

Evidently, the inorganic constituents of the infusions are not responsible for the excystment-inducing activity. This is made particularly evident by incinerating a quantity of dried yeast-autolysate in a porcelain crucible until only the ash of inorganic constituents remained. A solution of this ash was then compared with a solution of the original material. The former had lost all its activity. This indicates very strongly that some combustible organic compound is the active material in the yeast preparation.

The experiments with solutions of various acidities indicate that a simple change in pH is insufficient to bring about excystment. This result is in direct contradiction to the statements of Koffmann (1924), who claimed that both encystment and excystment of

Colpoda species was primarily a function of the hydrogen ion concentration. His interpretation is evidently quite incorrect.

Hay infusions contain strongly surface-active materials. This may be qualitatively demonstrated by placing a speck of a concentrated hay extract on a clean water surface. With a low-power microscope the speck may be seen to tear apart and scatter over the surface, with explosive rapidity. One might expect, therefore, that excystment inducing activity is dependent upon surface activity in such a way that any strongly active material in low concentration should induce excystment. The experiments with various surface-active substances, however, indicate that this is certainly not true in the concentrations used. There can be no doubt but that this class of substances does affect the cysts. If the cysts are examined under high magnification immediately before and after application of any of these substances, a stimulation evidenced by increased or decreased rate of pulsation of the contractile vacuole and of cyclosis of the protoplasm will be observed. But not every stimulus gives rise to the excystment response. Apparently, only very special stimuli induce this particular, physiologically important response.

Foods, waste products of metabolism, and materials affecting oxidation-reduction potentials, all of which might conceivably be of importance in nature as stimuli, are completely inactive.

These combined results indicate that many of the organic substances contained in infusions certainly are without any excystment-inducing activity. Yet, as the experiments with incinerated yeast infusion show, some organic material is certainly the active agent. One is led to the hypothesis that excystment in *Colpoda cucullus*, under the conditions of these experiments, is induced by some specific substance or by a very restricted group of substances possessing specific properties. This is the more curious since the specific agent appears to be generally extractable from plant and animal tissues.

This hypothetic material is active in very low concentrations. Consider the maximum amount of active material which is probably present in the most dilute ($\frac{1}{10}$ per cent) solution of hay extract that will just induce excystment. Since the hay extract is composed largely of carbohydrate, protein, fat, and substances many of which the foregoing experiments have indicated to be inactive, certainly the active agent composes not more than 1 per cent of the solid material. From these estimates we may calculate that excystment is induced by one part of active material in from ten to one hundred million parts of solution.

SUMMARY

1. A technique is described for the preparation of the cysts of *Colpoda cucullus* for excystment experiments. The cysts so prepared are relatively uniform in their responsiveness to excystment stimuli.

2. A method of studying the stimuli which induce excystment is described which provides for adequate control experiments.

3. All plant and animal extracts studied are active in inducing excystment of *Colpoda* from its resting cysts.

4. Pure chemicals, whether inorganic or organic, appear to be completely inactive.

5. Simple change in pH does not of itself induce excystment.

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THE INFLUENCE OF CARBON DIOXIDE UPON THE RESPIRATION OF MUSCLE

(One figure)

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THIS investigation was undertaken to determine whether or not the depression of respiration that has been shown to occur in nerve under the influence of carbon dioxide (Root, 1932) also occurs in skeletal muscle. Moreover, in view of Lipmann and Meyerhof's study (1930) of the influence of carbon dioxide upon the acid-base changes in muscle, such a study should be of especial interest.

EXPERIMENTAL METHOD

Rana pipiens was pithed, and the gastrocnemii muscles cut as close as possible to their connections with the bones. The muscles were gently touched to filter paper to remove any adherent moisture, and quickly weighed on a torsion balance. They were placed on the stimulating electrodes of tonometers which have been described by Root (1932). The tonometers were then put in a constant-temperature water bath, and gas mixtures which had been stored in gasometers were flushed through for about 1 hour. A sample of the gas, after it had passed through the tonometers and immediately before the tonometer was closed, was collected in a sampling tube similar to that used by Bazett and Sribyatta (1928). Analysis of this sample gave the initial gas tensions within the tonometer.

At the end of test periods varying from 4¾ to 21 hours, the tonometers were removed from the water bath, and a sample of the contained gas was withdrawn in the special collector and set aside for later analysis. In some of the experiments the irritability of the muscles was tested by the threshold method.

The gas samples were analyzed by means of a Haldane-Henderson analyzer with a nitrogen side tube, the principle being that employed in Bazett's (1928) modification. The percentage of difference in the initial and final oxygen analyses varied from 0.60 to 3.82. Meyerhof (1930) states that after the first 2 or 3 hours the respiration of isolated frog muscle remains constant for 24 hours. In no experiment has the measurement of respiration exceeded this limit.

The muscles, although perhaps slightly injured by their removal from the bones, seemed always in good resting condition and never showed any shrinking or spontaneous twitches.

The total oxygen and carbon dioxide present at the beginning and at the end of the experiment were calculated, the usual correction for barometer, temperature, and water vapor being applied. No correction for nitrogen was made. It is difficult to see how this can be done, because changes in the percentage of nitrogen due to the error of analysis may at high oxygen tensions result in corrections greater than the respiratory exchange of the tissue. This has been pointed out by Briggs (1920) and by Hill and his co-workers (1924).

RESULTS AND DISCUSSION

The results obtained in eleven experiments on the resting respiration of right and left gastrocnemii muscles are given in Table I. It will be seen that the maximum difference in the rate of oxygen consumption in any one experiment is 13 per cent. The carbon dioxide values showed even a greater fluctuation, though great care was exercised to avoid pressure changes within the tonometers in withdrawing the gas samples. The differences in the rates of carbon dioxide production were as great as 22 per cent in one experiment. The average difference in the rate of oxygen consumption of the matched gastrocnemii muscles of Table I is 1 per cent. The average difference for the rate of carbon dioxide production is 3 per cent.

Forty-one experiments were carried out in an investigation of the respiratory exchanges of resting gastrocnemii muscles at 22.0 ± 0.2° C. (Table II). The rate of oxygen consumption varied from 23.7 to 86.7 cu. mm. per gram of fresh weight per hour, with an average value of 46.8 cu. mm. At this temperature Meyerhof (1930) reports a range of 25–55 cu. mm. of oxygen consumption per gram and hour, with an average of 35 cu. mm. Fenn (1927) found an average value of 153 cu. mm. oxygen consumption per gram per hour. The more recent data from Fenn's laboratory (Fenn and Latchford, 1932) indicates values in agreement with those reported here. Unfortunately, the excitability of most of the muscles of Table II was not determined. It is possible that some of the muscles whose respiration was high were inexcitable.

TABLE I

THE RESPIRATORY EXCHANGE OF PAIRED MUSCLES OF *Rana pipiens*

Exp. No.	Date 1931	Wt. (Mg.)	Temperature (°C.)	O_2 Tension End Exp. (Mm. Hg)	Duration Exp. (Hr.)	O_2 Consumption (Cu. Mm./G./Hr.)	CO_2 Production (Cu. Mm./G./Hr.)	RQ	O_2 Ratio (B/A)	CO_2 Ratio (B/A)
III A	2-9	439	22±0.5	713.8	5.20	31.4	33.1	1.05	1.07	1.13
III B	2-9	427	22±0.5	713.5	4.86	33.6	37.3	1.11		
XI A	2-26	500 674	15±0.5	704.6	6.10	15.2	15.2	1.00	1.13	1.14
XI B	2-26	513 669	15±0.5	703.1	6.56	17.1	17.4	1.02		
XII A	2-27	650	15±0.5	713.2	16.63	20.5	22.1	1.08	0.96	0.98
XII B	2-27	642	15±0.5	713.9	16.55	19.6	21.6	1.10		
XIII A	2-28	629	15±0.5	710.5	17.90	20.1	19.9	0.99	1.00	1.17
XIII B	2-28	629	15±0.5	710.5	17.80	20.1	23.3	1.16		
XV A	3-3	617	15±0.2	620.0	18.06	16.7	15.5	0.93	0.96	0.95
XV B	3-3	610	15±0.2	620.9	17.55	16.1	14.7	0.91		
XVI A	3-4	508	15±0.2	673.8	17.42	14.6	15.3	1.09	0.89	0.78
XVI B	3-4	508	15±0.2	674.1	18.11	13.0	12.0	0.92		
XXIV A	3-14	452	15±0.5	689.0	20.10	15.7	12.7	0.81	0.97	0.80
XXIV B	3-14	449	15±0.5	689.1	20.15	15.3	10.2	0.67		
XXVI A	3-21	471	22±0.5	626.5	12.33	44.1	......	....	0.95	.....
XXVI B	3-21	441	22±0.5	627.6	12.26	41.9	44.9	1.07		
XXXII A	3-28	519	22±0.2	652.8	20.90	45.1	43.9	0.97	0.94	0.84
XXXII B	3-28	515	22±0.2	601.4	20.81	42.2	37.0	0.88		
XIX A	3-7	490	15±0.5	696.2	20.58	12.3	12.5	1.02	1.04	0.98
XIX B	3-7	499	15±0.5	682.9	20.66	12.8	12.3	0.96		
XX A	3-9	659	15±0.5	639.3	16.76	23.6	23.1	0.98	1.02	0.89
XX B	3-9	606	15±0.5	605.9	17.01	24.1	20.5	0.85		
Average									0.99	0.97

TABLE II

THE OXYGEN CONSUMPTION OF RESTING CASTROCNEMIUS MUSCLE AT HIGH OXYGEN TENSIONS (*Rana pipiens*)
22.0° ± 0.2 C.

Exp. No.	Date 1932	Wt. (Mg.)	O_2 Tension End Exp. (Mm. Hg)	Exp. Time (Hr.)	O_2 Consumption (Cu. Mm./G./Hr.)	CO_2 Production (Cu. Mm./G./Hr.)	Apparent RQ
*1	2-9	439	714	5.20	31.4	33.1	1.05
*2	2-9	427	714	4.86	33.6	37.3	1.11
3	3-3	298	689	8.79	86.7	33.8	0.39
4	3-18	645	688	5.36	36.1	36.5	1.01
*5	3-23	575	686	17.78	34.7	31.4	0.90
6	3-23	610	681	4.73	29.5	26.6	0.90
7	3-16	520	667	4.75	31.4	20.7	0.66
*8	3-25	459	667	8.71	37.9	37.6	0.99
9	3-24	480	655	5.51	67.2	88.0	1.31
*10	3-28	519	653	20.90	45.1	43.9	0.97
11	3-3	395	649	10.45	46.7	45.3	0.97
12	3-23	545	642	4.71	38.7	37.2	0.96
13	3-24	630	640	5.71	71.9	67.9	0.94
*14	3-23	638	639	10.26	23.7	16.9	0.71
15	3-22	515	639	4.66	32.3	34.9	1.08
*16	3-1	750	639	9.40	43.8	25.4	0.58
*17	3-21	441	628	12.26	41.9	44.9	1.07
*18	3-29	497	625	8.73	32.7	24.9	0.76
19	3-18	575	618	6.00	30.6	30.9	1.01
20	3-9	460	609	4.39	67.9	51.6	0.76
*21	4-13	296	607	16.13	47.0	40.5	0.95
22	3-22	600	602	5.55	48.3	46.9	0.97
*23	3-28	515	601	20.81	42.2	37.0	0.88
24	3-10	520	598	4.51	84.7	53.4	0.66
25	3-21	490	598	4.63	70.0	78.4	1.12
26	3-22	475	598	4.70	31.1	28.3	0.91
27	3-4	550	572	9.41	54.4	41.0	0.75
28	3-9	495	571	5.63	43.9	39.5	0.90
29	3-23	610	557	5.10	40.8	32.6	0.80
30	3-21	495	558	4.52	39.0	44.9	1.15
31	3-11	440	543	4.63	61.7	61.7	1.00
32	3-14	575	542	4.89	51.1	33.7	0.66
*33	4-13	300	536	15.88	47.0	40.5	0.85
34	3-22	625	534	6.00	38.8	41.1	1.06
35	3-23	555	516	5.17	50.8	47.2	0.93
36	3-16	510	511	4.80	45.3	34.4	0.75
37	3-1	485	482	11.26	55.3	27.2	0.49
38	3-24	490	469	5.59	45.3	31.2	0.69
39	3-8	675	445	5.73	64.8	33.6	0.52
40	3-24	640	458	6.16	64.9	56.2	0.87
41	3-14	435	423	5.20	29.2	37.1	1.25
Average					46.8	40.1	0.86

* 1931.

The rate of carbon dioxide production fluctuates between 16.9 and 78.4 cu. mm. per gram per hour. The average is 40.1 cu. mm. The apparent respiratory quotients which are quite variable range from 0.39 to 1.25, with an average of 0.86.

The duration of the experiments (4.39–20.90 hours) and the weights of the muscles (298–750 mg.) are without influence upon the rate of oxygen consumption.

Fenn (1930) confirmed Dulière and Horton's (1929) observations upon non-irritable muscle and showed that such muscles consume oxygen at 1.7 times the rate of irritable muscles. In nine experiments on matched muscles (Table III), one muscle showed a much higher rate of respiratory exchange which varied from 1.34 to 2.40 times the companion muscle. The carbon dioxide output in the rapidly metabolizing muscle was from 1.05 to 2.58 times that of its mate. The average increase is 1.75 for oxygen and 1.76 for carbon dioxide. The averages agree well with Fenn's value of 1.7. It is interesting to note that the apparent respiratory quotient for the excess metabolism is approximately 1.0. Only 40 per cent of the muscles which gave the high respiratory exchanges were completely inexcitable. This may mean that the onset of the increased rate of respiratory exchange precedes the appearance of complete inexcitability.

The thickness of gastrocnemii muscles of 300–750 mg. weight raises the question of whether or not the oxygen tension is sufficiently high to supply the fibers at the center of the tissue. Because of the irregular shape of an isolated muscle, it is difficult to calculate the true thickness at the large end. It is stated by Meyerhof and Schulz (1927) that a gastrocnemius muscle of 0.9–1.0 gram weight and an oxygen consumption of 30 cu. mm. per gram per hour will not suffer from anoxemia in an atmosphere of oxygen. This would indicate that at least some of the muscles of Table II were adequately supplied with oxygen. The influence of oxygen tension upon the rate of oxygen consumption has been tested directly in three experiments (Table IV). The oxygen tension was decreased in one of each pair of matched muscles. In no case was a significant change in the rate of oxygen consumption noted. This point has been further tested by placing the experiments of Table II in eight groups of five experiments each and averaging the rates of oxygen consumption (Table V). It will be noted that there is no significant depression of oxygen consumption when the oxygen tension is decreased. In fact, the two groups whose oxygen tension was lowest show a slightly higher rate of oxygen consumption. It may be concluded that an oxygen tension of 425 mm. Hg or higher is sufficient to adequately supply resting frog gastrocnemii muscles of 300–750 mg. weight.

TABLE III

THE RESPIRATORY EXCHANGE OF "INEXCITABLE" GASTROCNEMIUS MUSCLE OF *Rana pipiens*

Exp. No.	Date 1931	Wt. (Mg.)	Temperature (°C.)	O_2 Tension End Exp. (Mm. Hg)	Duration Exp. (Hr.)	O_2 Consumption (Cu. Mm./G./Hr.)	CO_2 Production (Cu. Mm./G./Hr.)	RQ	O_2 Ratio (B/A)	CO_2 Ratio (B/A)
II A	2–7	597	22±0.5	727.1	4.78	41.7	42.1	1.05	1.61	1.68
II B	2–7	647	22±0.5	720.6	4.75	67.2	71.0	1.11		
IV A	2–11	664	15±0.5	674.5	19.25	19.0	18.8	0.99	1.39	1.38
IV B	2–11	578	15±0.5	681.9	18.88	26.5	26.1	0.99		
X A	2–25	967	15±0.5	709.2	11.41	15.5	14.3	0.92	2.40	2.58
X B	2–25	965	15±0.5	694.6	11.35	37.3	37.0	0.99		
XIV A	3–2	315	15±0.5	641.2	18.15	15.3	15.3	1.00	2.08	1.58
XIV B	3–2	318	15±0.5	639.7	18.05	31.9	24.2	0.76		
XVIII A	3–6	615	15±0.5	641.4	20.00	25.2	25.1	1.00	1.55	1.59
XVIII	3–6	625	15±0.5	637.3	20.06	39.2	40.0	1.02		
XXVIII A	3–24	638	22±0.2	686.4	17.78	34.7	31.4	0.90	1.34	1.57
XXVIII B	3–24	650	22±0.2	681.0	17.63	46.4	49.5	1.07		
XXIX A	3–25	459	22±0.2	667.3	8.71	37.9	37.6	0.99	2.19	2.23
XXIX B	3–25	461	22±0.2	678.2	8.60	83.0	84.7	1.02		
XXXI A	3–27	580	22±0.2	680.8	10.23	32.0	28.9	0.90	1.46	1.05
XXXI B	3–27	577	22±0.2	686.1	10.13	46.9	30.5	0.65		
XXXIII A	3–29	497	22±0.2	625.0	8.73	32.7	24.9	0.76	1.78	2.26
XXXIII B	3–29	505	22±0.2	593.9	8.85	58.2	56.7	0.98		
Average									1.75	1.76

The influence of carbon dioxide upon the rate of oxygen consumption in frog resting gastrocnemius muscle has been studied in nineteen experiments (Table VI). The rate of oxygen consumption of the matched control muscle is assumed to be 100. The percentage of variation from this value is calculated for the muscle exposed to carbon dioxide and plotted as the ordinate (Fig. 1). The carbon dioxide tension to which the experimental muscle is exposed is indicated along the abscissa. It will be observed that there is a pro-

TABLE IV

THE INFLUENCE OF OXYGEN TENSION UPON OXYGEN CONSUMPTION OF RESTING GASTROCNEMIUS MUSCLE

22.0° C.

Exp. No.	Date 1932	Wt. (Mg.)	Duration Exp. (Hr.)	O_2 Tension (Mm. Hg)	CO_2 Tension (Mm. Hg)	O_2 Consumption (Cu. Mm./G./Hr.)	CO_2 Production (Cu. Mm./G./Hr.)	Apparent RQ	O_2 Ratio (B/A)	CO_2 Ratio (B/A)
*1 A.....	3-28	519	20.90	681-653	1.7-29.3	45.1	43.9	0.97	0.93	0.84
*1 B.....	3-28	515	20.81	628-602	1.4-24.9	42.2	37.0	0.88		
2 A.....	3-22	515	4.66	640-635	1.9-6.7	32.3	34.9	1.08	0.96	0.81
2 B.....	3-22	475	4.70	603-598	2.3-5.9	31.1	28.3	0.91		
3 A.....	3-24	630	5.71	652-640	1.1-13.6	71.9	67.9	0.94	0.90	0.83
3 B.....	3-24	640	6.16	472-458	1.0-13.0	64.9	56.2	0.87		

* 1931.

TABLE V

THE INFLUENCE OF OXYGEN TENSION UPON OXYGEN CONSUMPTION OF RESTING GASTROCNEMII MUSCLES (*Rana pipiens*)

Group No.	O_2 Tensions (Mm. Hg)	O_2 Consumption (Cu. Mm./G./Hr.)	CO_2 Production (Cu. Mm./G./Hr.)	Apparent RQ
1..............	714-686	44.5	34.4	0.77
2..............	681-653	42.2	43.3	1.02
3..............	649-639	42.7	40.4	0.95
4..............	639-609	43.4	35.5	0.82
5..............	607-598	58.4	51.2	0.88
6..............	598-558	41.8	37.2	0.89
7..............	543-516	49.9	44.8	0.90
*8..............	511-423	50.8	36.6	0.72
Range and means..	714-423	46.8	40.1	0.86

* Each group contains 5 experiments except group 8, which contains 6.

TABLE VI

THE INFLUENCE OF CARBON DIOXIDE UPON THE RATE OF OXYGEN CONSUMPTION OF RESTING GASTROCNEMIUS MUSCLE (*Rana pipiens*)

T = 22.0° ± 0.2° C. Equilibration time, 1 hour

Exp. No.	Date 1932	Wt. (Mg.)	Duration Exp. (Hr.)	O_2 Tension (Mm. Hg)	CO_2 Tension (Mm. Hg)	O_2 Consumption (Cu. Mm./G./Hr.)	CO_2 Production (Cu. Mm./G./Hr.)	Apparent RQ	O_2 Ratio (B/A)	Ratio CO_2
*†‡ 1 A. .	3–7	490	20.58	703–696	0.8– 7.9	12.3	12.5	1.02	1.04	0.88
1 B. .	3–7	499	20.66	689–684	19.6– 25.6	12.8	12.3	0.96		
*†§ 2 A. .	3–9	606	17.01	620–606	0.7– 12.7	24.1	20.4	0.85	0.98	1.00
2 B. .	3–9	659	16.76	654–640	22.6– 37.1	23.6	23.1	0.98		
3 A. .	3–9	495	5.63	577–571	3.0– 8.6	43.9	39.5	0.90	0.72	0.92
3 B. .	3–9	505	5.23	623–619	43.8– 48.9	31.9	36.7	1.15		
4 A. .	3–9	460	4.39	617–609	3.0– 9.0	67.9	51.6	0.76	0.84	0.72
4 B. .	3–9	490	4.31	648–641	59.6– 64.2	57.2	37.2	0.65		
5 A. .	3–18	575	6.00	624–618	0.7– 6.9	30.6	30.9	1.01	0.82	0.90
5 B. .	3–18	535	5.80	587–582	61.1– 65.9	25.2	27.7	1.10		
6 A. .	3–18	645	5.36	694–688	0.7– 7.6	36.1	36.5	1.01	0.54	0.45
6 B. .	3–18	625	5.46	640–637	64.0– 67.8	19.6	16.3	0.83		
7 A. .	3–11	440	4.63	550–543	1.1– 7.5	61.7	61.7	1.00	0.40	0.42
7 B. .	3–11	445	4.90	598–595	67.6– 70.6	24.6	25.8	1.05		
8 A. .	3–4	550	9.41	587–572	0.5– 12.1	54.4	41.0	0.75	0.32	0.45
8 B. .	3–4	610	9.66	594–585	68.2– 77.6	17.3	18.5	1.07		
9 A. .	3–10	520	4.51	609–598	0.9– 8.0	84.7	53.4	0.63	0.62	0.55
9 B. .	3–10	520	4.61	629–621	75.2– 80.1	52.8	29.6	0.56		
10 A. .	3–3	298	8.79	702–689	1.3– 6.7	86.7	33.8	0.39	0.88	0.81
10 B. .	3–3	288	8.70	633–622	75.6– 78.9	74.4	27.5	0.36		
11 A. .	3–1	750	9.40	653–639	3.3– 13.8	43.8	25.4	0.58	0.65	0.67
11 B. .	3–1	750	9.50	606–595	90.8– 97.4	28.5	16.9	0.59		
*‡12 A. .	4–13	296	16.13	618–607	1.4– 13.5	47.0	40.5	0.95	0.40	0.59
12 B. .	4–13	300	15.88	540–536	93.4– 99.8	18.8	24.0	1.28		
13 A. .	3–3	395	10.45	659–649	3.2– 11.3	46.7	45.3	0.97	0.53	1.46
13 B. .	3–3	400	10.39	569–563	97.4–113.1	24.9	66.7	2.68		

* 1931.

† 15.0° C.

‡ Equilibrium time, 3½ hours.

§ Equilibrium time, 5½ hours.

TABLE VI—*Continued*

Exp. No.	Date 1932	Wt. (Mg.)	Duration Exp. (Hr.)	O_2 Tension (Mm. Hg)	CO_2 Tension (Mm. Hg)	O_2 Consumption (Cu. Mm./G./Hr.)	CO_2 Production (Cu. Mm./G./Hr.)	Apparent RQ	O_2 Ratio (B/A)	Ratio CO_2
14 A..	3–16	520	4.75	672–667	1.7– 4.7	31.4	20.7	0.66	0.63	1.16
14 B..	3–16	535	4.83	593–590	118.3–121.8	20.0	24.0	1.20		
15 A..	3–16	510	4.80	518–511	0.6– 6.0	45.3	34.4	0.75	0.22	0.70
15 B..	3–16	505	5.40	530–528	121.1–124.7	9.8	24.0	2.45		
16 A..	3–1	485	11.26	495–482	4.1– 13.2	55.3	27.2	0.49	0.67	0.23
16 B..	3–1	498	11.31	614–596	125.6–127.5	36.8	6.3	0.17		
17 A..	3–8	675	5.73	458–445	1.5– 8.6	64.8	33.6	0.52	0.64	0.25
17 B..	3–8	690	5.83	500–491	125.5–127.8	41.3	10.7	0.26		
18 A..	3–14	435	5.20	426–423	1.2– 5.3	29.2	37.1	1.25	0.34	0.85
18 B..	3–14	420	5.66	490–489	133.0–137.1	10.2	37.7	3.11		
19 A..	3–14	575	4.89	548–542	1.8– 6.5	51.1	33.7	0.66	0.57	1.07
19 B..	3–14	550	5.03	544–540	152.8–157.8	29.2	35.9	1.23		

gressive decrease in the rate of oxygen consumption which begins at 40 mm. Hg carbon dioxide tension and amounts to 50 per cent of the control value at 93 mm. Hg. Further increases in the carbon dioxide tension depress the rate of oxygen consumption proportionately less. It is interesting to note that the depression in oxygen consumption is not affected by variations in duration of the experiment. The control experiments of Table I have been inserted in Figure 1.

In all the experiments of Table VI, the oxygen tension was kept above 423 mm. Hg. From the data of Tables IV and V the tensions employed would appear adequate; so it is unlikely that the results here reported were due to a lack of oxygen. It is, of course, possible that the oxygen tension at which the effects of oxygen deficiency appear, may not be the same in the presence of high tensions of carbon dioxide as in the relative absence of this gas. If the foregoing experiments had been carried out in the region of oxygen deficiency, however, even slight increases in the oxygen tension would have resulted in an increased rate of oxygen consumption. That this is not the case is shown by a comparison of experiments 4 and 5, and experiments 14 and 16 (Table VII), which shows that, when the oxygen-tension difference in paired experiments is reversed, the differences

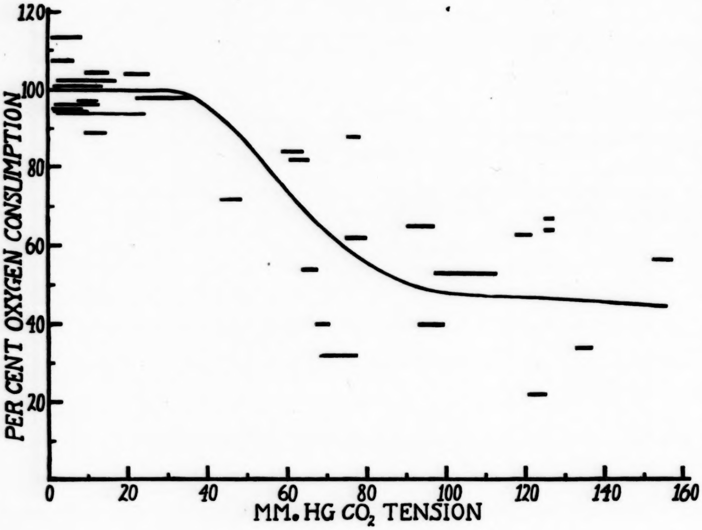

FIG. 1.—The influence of carbon dioxide upon the rate of oxygen consumption of resting skeletal muscle (*Rana pipiens*). Abscissa = mm. Hg carbon dioxide tension; ordinate = percentage of oxygen consumption.

TABLE VII

THE INFLUENCE OF OXYGEN TENSION UPON THE RATE OF OXYGEN CONSUMPTION IN THE PRESENCE OF CARBON DIOXIDE

Exp. No.	O_2 Tension (Mm. Hg)	CO_2 Tension (Mm. Hg)	O_2 Consumption (Cu. Mm./G./Hr.)	O_2 Ratio (B/A)
4 A	617–609	3.0– 9.0	67.9	0.84
4 B	648–641	59.6– 64.2	57.2	
5 A	624–618	0.7– 6.9	30.6	0.82
5 B	587–582	61.1– 65.9	25.2	
14 A	672–667	1.7– 4.7	31.4	0.63
14 B	593–590	118.3–121.8	20.0	
16 A	495–482	4.1– 13.2	55.3	0.67
16 B	614–596	125.6–127.5	36.8	

in the rates of oxygen consumption lie within the experimental error of the method. The carbon dioxide tensions in the experiments compared do not differ by enough to affect appreciably the rates of the oxygen consumption.

The influence of carbon dioxide upon the rate of carbon dioxide production of skeletal muscle is exceedingly variable. The carbon dioxide tension, the duration of the equilibrium period, the metabolism of the tissue, and possible errors in technique appear as important factors. Since Lipmann and Meyerhof's (1930) work indicated an increased acid binding power of frog's skeletal muscle in the presence of carbon dioxide, a decrease in the apparent carbon dioxide production would be expected. While this may be shown at lower tensions, carbon dioxide concentrations greater than 90 mm. Hg indicated increased carbon dioxide production in six out of eight experiments. It may be that high carbon dioxide tensions injure the cells and liberate fixed acid.

The present experiments indicate that the decrease in the rate of oxygen consumption of the dogfish lateral line nerve in the presence of carbon dioxide (Root, 1932) is not peculiar to this tissue but also occurs in frog's skeletal muscle. In this connection it is interesting to note that the oxygen consumption of frog's heart (Clark and White, 1928) and of the fertilized eggs of Arbacia (Root, 1930) is suppressed by carbon dioxide.

Necheles and Gerard's (1930) statement that the influence of carbon dioxide upon nerve activity can be summarized in terms of slowed chemical reactions is in agreement with the slowed speed of oxidative recovery found in muscle exposed to carbon dioxide (Hartree and Hill, 1924). This view is also in accord with the results obtained by Bozler (1930) upon the retractor muscle of the snail, and with the data reported above.

SUMMARY

1. The respiratory exchanges of the gastrocnemius muscle of *Rana pipiens* have been studied by the method of gas analysis.

2. The respiratory exchanges of matched muscles have been measured and found to agree within 15 per cent.

3. The increased rate of respiration of inexcitable muscle reported

by an earlier investigator is confirmed. The average increase is 1.75 for oxygen and 1.76 for carbon dioxide.

4. The rate of oxygen consumption of frog gastrocnemius muscle at 22.0° C. ranges between 23.7 and 86.7 cu. mm. per gram of fresh weight per hour, with an average of 46.8 cu. mm. The rate of carbon dioxide production varies between 16.9 and 78.4 cu. mm. per gram per hour. The average is 40.1. The apparent (uncorrected for free or combined carbon dioxide in tissues) respiratory quotients average 0.86.

5. The respiration of frog gastrocnemii muscles of 300–750 mg. weight is unchanged when the oxygen tension is decreased to 425 mm. Hg (22.0° C.).

6. The rate of oxygen consumption in the presence of carbon dioxide decreases progressively up to 93 mm. Hg carbon dioxide tension, at which point it is 50 per cent of the control value. Further increases in the carbon dioxide tension up to 160 mm. Hg depress the rate of oxygen consumption proportionately less. In the presence of carbon dioxide, the apparent carbon dioxide production is exceedingly variable.

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THE EFFECT OF HYPERTONIC SOLUTIONS ON THE OXYGEN CONSUMPTION OF A DEVELOPING EGG (ORTHOPTERA)

(Six figures)

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THE relationship between the water content and oxygen consumption of tissues, as well as of entire organisms, is of considerable interest in lieu of the apparently conflicting data reported by various investigators (Warburg, 1908, 1909; Meyerhoff, 1911; Loeb and Wasteneys, 1913; Inman, 1921; Caldwell, 1925, 1931; Hess, 1930; Buchanan, 1930, 1931; Beadle, 1931; Duryee, 1932; and others). Loss of water in many forms has been found to be accompanied by increased oxygen consumption, while quite the opposite effects have been noted for other forms. Additions of water have likewise been reported to have produced both increases and decreases in the oxygen consumption in a variety of organisms. In the present paper, results of experiments carried out on the effects of loss of water on the oxygen consumption of the egg of the common grasshopper, *Melanoplus differentialis*, are presented.

The eggs of the grasshopper have been found extremely favorable material for metabolism studies (Bodine, 1929). All eggs used in the present experiments were in the same morphological stage of development—the stage designated by Slifer (1932) as the "3-week stage." It is of importance to note that eggs in different stages of embryonic development react in very different ways to loss of water (Bodine, 1929). As pointed out by Slifer (1932), the chorion, or outer covering of the egg, can easily be removed at the 3-week stage; and the embryo is thus easily visible through the chitinous cuticle inclosing it. This chitinous cuticle, as will be shown in detail below, is markedly semipermeable in nature; and it is upon this property that the present results are based. A point of further interest is the fact that developing grasshopper eggs can be kept under water as readily as in air with no appreciable effects other than a slight retardation in the rate of development. This slowing-down of development is undoubtedly associated with marked reductions in oxygen supply as well as with accumulations of carbon dioxide. The animal, at the hatching stage, is not able to completely free itself and emerge from the egg under water after the chitinous cuticle is broken.

Oxygen determinations have been carried out at constant temperature of 28° C., using the Krogh manometer (Bodine, 1929); and in each experiment groups of eggs ranging in number from 5 to 25 have been used at one time. All determinations were carried out over a period of 3 hours when the eggs, free from solutions, were suspended in the manometer vessels. All eggs were kept at room temperature in covered Syracuse watch glasses containing 10–15 cc. of solution. One hundred to 150 eggs were placed in each watch glass, and solutions were changed daily.

Changes in the water content of the eggs were brought about by subjecting them to various strengths of a modified Ringer solution (Belar, 1929). This solution, which has been found practically isotonic with the egg, and which has been designated throughout this paper as "normal Ringer solution," has the following composition: 0.9 per cent NaCl, 0.05 per cent KCl, 0.05 per cent $CaCl_2$, 0.05 per cent $NaHCO_3$. Solutions both with and without $NaHCO_3$ have been used, and no significant differences in results have been obtained. Hypertonic solutions, made by increasing the concentration of the various constituents, change the water content of eggs in a rather striking fashion. The question of rate of water loss and related phenomena will be fully discussed in a subsequent communication from this laboratory by Dr. E. Slifer.

EXPERIMENTAL RESULTS

I. *Oxygen consumption of eggs in distilled water, tap water, and normal Ringer solution.*—Eggs, in 3-week stage of development, were put and kept in the foregoing solutions, and their rates of oxygen consumption determined daily up to the time of hatching. Figure 1 shows graphically results typical of such an experiment. From an inspection of these curves it will be noted that no significant differences in the rates of oxygen consumption of the eggs in the various solutions are indicated. Apparently, the egg is in some way able to regulate its water relations when in isotonic or hypotonic solutions. Weights and appearances of embryos remain quite normal throughout the period of development.

II. *Oxygen consumption of eggs in hypertonic solutions.*—Various strengths of hypertonic normal Ringer solution have been used, and their effects on the rates of oxygen consumption are found to be more or less directly proportional to their osmotic pressures.

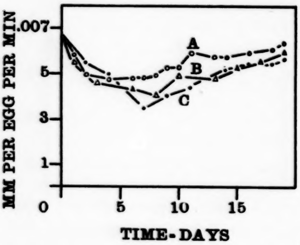

FIG. 1.—Showing rates of oxygen consumption for eggs of *Melanoplus differentialis* in distilled water, tap water, and normal Ringer solution. Eggs at room temperature, 28°± 1°C.; oxygen determination at constant temperature of 28° C. Ordinates, millimeters change of manometer fluid per egg per minute (same manometer used throughout experiment; to convert readings into actual amounts of oxygen, results are multiplied by factor for the manometer in question). Abscissae, time in days indicated: *A* = eggs in normal Ringer solution; *B* = eggs in distilled water; *C* = eggs in tap water. For further description see text.

(1) 2 × normal Ringer solution:

A slightly hypertonic solution (2 N) has little effect on the oxygen consumption of the egg for a relatively long period of time (2 weeks at 27° C.), and even then its action is quite slow. In Figure 2 there is graphically shown results typical of such an experiment. From an examination of these it will be seen that no significant effect on the egg occurs until after some 15 days' exposure to the solution. At this time the rate of oxygen consumption drops below that for the controls in tap water. A rather gradual fall in the rate of oxygen consumption follows and reaches a minimum around the twenty-eighth to thirtieth day. Curiously, this minimum (at 27°) is more or less constant for this concentration (2 N) over rather long periods of time. In some cases it has been maintained for much over 2 weeks without seriously injuring the egg, as judged by subsequent development when returned to tap water. When eggs, treated with this concentration (2 N), are returned to tap water or isotonic Ringer solution, they quickly recover, as shown by their rapid increase in rate of oxygen consumption, and in a short time reach the hatching stage (Fig. 2). A point of some interest is the slow change in rate of oxygen consumption upon immersion in the 2 N solution as compared with the relatively faster rate of recovery in tap water. This would suggest a slower rate of water loss than of water entrance or recovery. Mortality of eggs in 2 N solution is very small, and recovery is practically 100 per cent.

A point of further note relates to the internal changes brought about by the loss of water through exposure to the hypertonic solutions. When the rate of oxygen consumption falls below that of the normal or control animal in tap water, further development of the egg practically ceases, and the embryo remains in this condition until water is subsequently readmitted. Cytological studies of embryos in these experiments show that water loss is both inter- and intra-cellular. Mitosis ceases, and considerable shrinkage of both nucleus and cytoplasm results. Yolk becomes reduced to small flakes, and the entire egg becomes distorted and shrunken and loses markedly in weight.

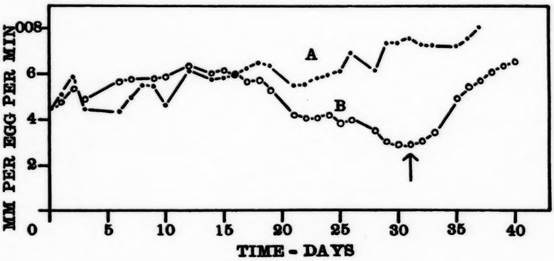

FIG. 2.—Showing rates of oxygen consumption for eggs of *Melanoplus differentialis* in 2 N Ringer solution as well as for their recovery when returned to tap water. Eggs kept at room temperature, 26° ± 1° C.; oxygen determinations at constant temperature of 28° C. Ordinates, millimeters change of manometer fluid per egg per minute (see legend under Fig. 1). Abscissae, time in days indicated: *A* = control eggs in tap water; *B* = eggs in 2 N Ringer solution. Arrow indicates time experimental eggs were transferred to tap water. For further description see text.

(2) 4 × normal Ringer solution:

With increase in osmotic pressure of the solution, there is also a marked increase in the relative rate of change in the reaction of the egg. In Figure 3 there is graphically shown results typical of the effects on the rate of oxygen consumption of an exposure to a 4 N Ringer solution, and it will be noted that for a short period of approximately a week (at 27° C.) no significant changes in rate of oxygen consumption over the control eggs are noted. This, it will be recalled, is much shorter a period than for the 2 N solution, where the change was only apparent after approximately 2 weeks' exposure. A gradual decrease in rate of oxygen consumption for the egg in the 4 N solution then follows, down to a minimum value. This minimum is not only relatively lower than that reached in the 2 N solution, but it is also reached in relatively shorter periods of exposure to the solution. Such a difference is usually, but not invariably, found, since considerable variation in final end-points for different lots of eggs exists. In the case of the eggs exposed to the 4N solution, when returned to tap water, a rather rapid increase in rate of oxygen consumption occurs. Complete recovery after exposure to the stronger hypertonic solutions depends to a remarkable degree upon the extent of the exposure, as well as upon the relative resistance of the particular lot of eggs. In the case indicated in Figures 3 and 4*C*, only partial recovery of rate of oxygen consumption was obtained, the degree of dehydration apparently being too great for complete

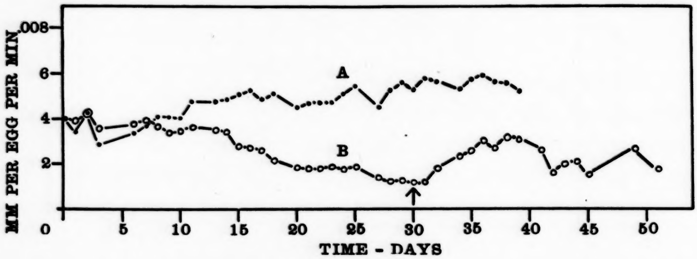

FIG. 3.—Showing rates of oxygen consumption for eggs of *Melanoplus differentialis* in 4 N Ringer solution as well as for their recovery when returned to tap water. Eggs kept at room temperature, 26° ± 1° C.; oxygen determinations at constant temperature of 28° C. Ordinates, millimeters change of manometer fluid per egg per minute (see legend under Fig. 1). Abscissae, time in days indicated: *A* = control eggs in tap water; *B* = eggs in 4 N Ringer solution. Arrow indicates time experimental eggs were transferred to tap water. For further description see text.

recovery. In other experiments, as indicated graphically in Figure 4, complete recovery has been obtained. From an examination of this figure it will be noted that in this case the depression in rate of oxygen consumption is rather large, amounting to approximately 60 to 65 per cent of the normal value. The morphological changes induced in the egg are such that the two sides of the egg seem approximated while the embryo and yolk are almost completely flattened out. Recovery, although rapid after the addition of water, does not seem to be as fast as for the weaker solution.

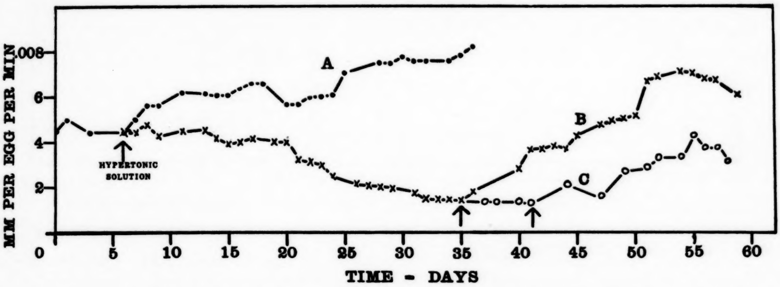

FIG. 4.—Showing rate of oxygen consumption for eggs of *Melanoplus differentialis* in 4 N Ringer solution as well as for their recovery when returned to tap water. Eggs kept at room temperature, 26° ± 1° C.; oxygen determinations at constant temperature of 28° C. Ordinates, millimeters change of manometer fluid per egg per minute (see legend under Fig. 1). Abscissae, time in days indicated: *A* = control eggs in tap water; *B* = eggs in 4 N Ringer solution; *C* = eggs in 4 N Ringer solution. Time of exposure to hypertonic solution indicated. Arrows indicate when experimental eggs were transferred to tap water. For further description see text.

(3) Markedly hypertonic Ringer solution: Various concentrations up to 10 N have been employed; and in all cases it has been found that, as the osmotic pressure increases, the rates of decrease in oxygen consumption increase, and that recovery becomes progressively more affected the longer the exposure. In Figure 5 are graphically shown the results, typical of an experiment with 10 N solution. It will be observed from this figure that a rapid decrease in the rate of oxygen consumption takes place down to a minimum value. Recovery, when the eggs are returned to tap water, takes place in eggs exposed to this concentration only for short periods, usually a week or 10 days. Beyond this time mortality is extremely high

(Fig. 5*C*). Rates of recovery are usually fairly rapid in the case of strongly hypertonic solutions.

Morphological changes in the egg are very marked in highly hypertonic solutions. The egg becomes completely flattened out and is extremely difficult to handle. Cytological studies of eggs dehydrated for periods of a week or over show the cells to be greatly distorted and shrunken. The nucleus stains as a dense circular mass, and no evidence of chromosomes or finer structures can be seen.

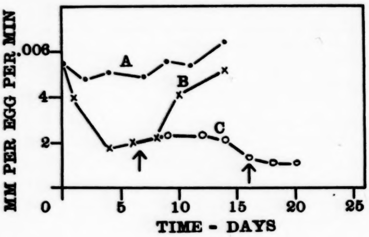

FIG. 5

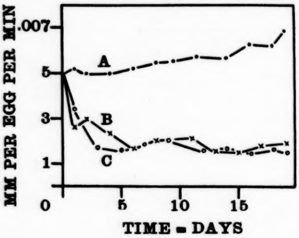

FIG. 6

FIG. 5.—Showing rates of oxygen consumption for eggs of *Melanoplus differentialis* in 10 N Ringer solution as well as for their recovery when returned to tap water. Eggs kept at room temperature, 30° ± 1° C.; oxygen determination at constant temperature of 28° C. Ordinates, millimeters change of manometer fluid per egg per minute (see legend under Fig. 1). Abscissae, time in days indicated: *A* = control eggs in tap water; *B* = eggs in 10 N Ringer solution; *C* = eggs in 10 N Ringer solution. Arrows indicate when experimental eggs were transferred to tap water. For further description see text.

FIG. 6.—Showing rates of oxygen consumption for eggs of *Melanoplus differentialis* in tap water, 5 N Ringer solution and 5 per cent NaCl solution. Eggs kept at room temperature, 30° ± 1° C.; oxygen determination at constant temperature of 28° C. Ordinates, millimeters change of manometer fluid per egg per minute (see legend under Fig. 1). Abscissae, time in days indicated: *A* = control eggs in tap water; *B* = eggs in 5 N Ringer solution; *C* = eggs in 5 per cent NaCl solution. For further description see text.

The effects of the hypertonic solutions are unquestionably largely, if not solely, osmotic. The chitinous cuticle of the eggs seems to act as a semipermeable membrane, since solutions of pure salts, like NaCl, of the same osmotic pressures as the hypertonic Ringer solutions produce the same relative effects upon the rates of oxygen consumption of the eggs. In Figure 6 there are shown results typical of the reaction of eggs to hypertonic NaCl solution. An inspection of this figure shows that no significant differences are indicated between the effect of a 5 N Ringer and a 5 per cent NaCl solution. Recovery in tap water after exposure to NaCl solution is practically similar to that for Ringer solution of the same osmotic pressure.

SUMMARY AND CONCLUSION

1. A study of the effect of hypertonic Ringer solutions on the rates of oxygen consumption of the developing egg of the grasshopper, *Melanoplus differentialis*, has been made.

2. Hypertonic solutions produce a decrease in the rates of oxygen consumption of the egg, the relative rates and amount of which being roughly proportional to the concentration or osmotic pressure of the solution employed.

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SEROLOGICAL STUDIES OF TISSUE ANTAGONISM IN THE DOMESTIC FOWL[1]

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THE possibility of selecting compatible donors in tissue transplantation by employing hemagglutination has been a subject of speculation and research since the time the blood groups in human beings were definitely established. Time has only increased the difference of opinion among the various investigators in the field.

Thus, Masson (1918) states that "blood grouping is just as important for good results in skin grafting as it is necessary in transfusions, and that it is governed by the same principles."

Schawan (1919) likewise believes that the same principle that applies to blood transfusions applies to skin transplantation. On the basis of his clinical experience with seventeen patients he concluded that

> isografts obtained from donors of the same blood group as the recipient or from Group IV[2] donors became permanent takes and grew almost if not equally as well as autografts. Isografts where the donor and recipient were of different groups did not remain as permanent growths except when Group IV skin was used or when the recipient was a member of Group I. Group I recipients grew permanent skin from donors of all of the four groups and apparently equally well. Group IV skin grew permanently on recipients of all groups, but only Group IV grafts and autografts remained as permanent takes on Group IV recipients.

Baldwin (1920) arrived at conclusions identical with those of Schawan.

Kubânyi (1924) also reports four cases in which the transplant in each case was taken from individuals whose red blood corpuscles were not agglutinated by the serum of the host.

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[1] This work was carried out at the University of Chicago during the writer's tenure of a National Research Council Fellowship. The expenses of this investigation were borne in part by the Committee for Research in Problems of Sex of the National Research Council and in part by the University of Chicago.

[2] Moss's classification.

In contrast to the results of the foregoing investigators are those of Underwood (1914), who transplanted skin tissue from seventeen different individuals to a single patient. All the foreign tissue was subsequently absorbed except that of the mother of the patient; that of the sister took to a degree. He attributed the reaction of the host to the foreign tissue to anaphylaxis. Similar difficulties were encountered by Holman (1924). Of a total of 319 small, deep pinch grafts from a mother to a son, both belonging to the same blood group, all were originally positive but were subsequently absorbed. In another patient, belonging to blood group IV, the skin from three individuals was transplanted, two belonging to blood group IV and one to blood group II. The grafts from each of the donors belonging to Group IV took but were subsequently absorbed; the grafts from the donor belonging to group II did not become a part of the host. On the other hand, all the autografts persisted and continued to spread until the entire denuded area was covered with epithelium. The results of Underwood and Holman are further supported by four cases reported by Eden (1922). Whereas all the autotransplants persisted, the homoiografts were all absorbed, even though they were from donors of the same blood group as the host. The view of Underwood and Holman that homoioplastic grafts will persist only in isolated cases regardless of blood groups is further supported by the results of Gatch (1911), Schöne (1916), Perthes (1917), McWilliams (1924), and Lexer (1925), the latter emphatically stating that the success of isografts may be relegated to mythology.

The only attempt, as far as the writer is aware, to correlate grafting and agglutination in lower animals is that of Ingebrigtsen (1912). Working with cats, he concluded on the basis of fourteen transplants of the carotid artery that the results of transplantation in the group in which there was interagglutination differed in no striking way from those obtained in the group in which there was no interagglutination.

It is evident from a review of the literature that the question of using agglutination as a means of detecting incompatibility between donor and host is still an open one. It was with the hopes of collecting critical evidence upon this subject that the experiments presented in this paper were undertaken.

The writer wishes to avail himself of this opportunity to express his appreciation to Dr. C. Philip Miller for his kindly counsel and criticism of this paper.

PREPARATION OF THE CHICKEN ANTI-ERYTHROCYTE SERUM

The monovalent serum was prepared by injecting at 4-day intervals 5 cc. of a 10 per cent suspension of washed chicken erythrocytes into the posterior auricular vein of a rabbit. A reasonably powerful serum was usually obtained after the fifth injection. The polyvalent serum was a composite of equal portions of the anti-chicken sera from seven different rabbits, two of which were each injected with the corpuscles of a single bird in the manner described above and five of which were injected simultaneously with red blood cells of two birds. Four-tenths of a cubic centimeter of washed packed corpuscles from each bird was injected in an approximately 10 per cent suspension of physiologic saline solution, and thereafter 3 cc. were injected at 4-day intervals. The sera of the rabbits used for experimental purposes all gave a strong preliminary agglutination titer in dilutions of 1:1000 within 2 hours at room temperature. The birds whose corpuscles were used in the preparation of the anti-chicken serum were selected from eight different strains of pure-bred Leghorns and three hybrids of Brown and White Leghorns. The three latter birds were previously demonstrated to have a large number of agglutinogens.

The agglutination tests were made with absorbed sera according to the method used by Landsteiner and Miller (1924). The sera were absorbed by adding to each portion of serum, diluted 1:15, washed blood sediment of each chicken to be tested. The mixture was allowed to stand for 1 hour at room temperature and overnight in the ice box. The amount of the washed blood corpuscles used for absorption varied from ten to twenty times the amount of pure serum. Because of the large amount of corpuscles necessary for complete absorption of the serum required in dilutions of 1:15, the serum was diluted 1:40 and 1:50 in two of the later experiments. In all the agglutination tests 0.3 cc. of the fluid was used to 1 drop of a 2.5 per cent suspension of red blood cells. The final readings were always made after the mixture was allowed to stand for 2 hours at room temperature, overnight or the equivalent length of time in the ice box, and then 1 hour at room temperature. Readings were usually also made after 2 hours at room temperature; but, owing to the fact that the reaction was incomplete, these have been omitted from the experimental data herewith presented. After the mixture stood at room temperature for 2 hours, overnight in the ice box, and then 1 hour at room temperature, very little change, if any, took place even if exposed to room temperature for several additional hours.

RELIABILITY OF THE SEROLOGICAL TECHNIQUE

That the reaction with an immune serum would be specific for any given individuals was to be expected on the basis of the work with artificially induced agglutinins by Landsteiner and Levine (1928*a*) with anti-human erythrocyte serum and with an artificial iso-agglutinating serum with chickens by Todd (1930*a*, 1930*b*). This was further demonstrated by the repetition after an interval of 20 days of an experiment, involving five absorbed sera and the corpuscles of eight different birds from as many different strains. The results in the two tests checked qualitatively and differed quantitatively to only a slight degree in but five combinations.

There was, however, a markedly different reaction between some of the same birds when another serum, sensitized to the corpuscles of another bird, was used; also, a different reaction was obtained between two sera from two different rabbits each injected with the corpuscles of the same bird. It therefore appears that the reaction depends not only upon the individuality of the bird but also upon the individuality of the rabbit injected. This also might have been expected from the work of Landsteiner and Levine (1928*a*) and Todd (1930*a*) and others.

It is, of course, realized that not even the polyvalent serum was sufficiently sensitive to bring out all the differences in all the individuals tested. The significance of the test must therefore be evaluated on the extent of the test between any two particular individuals concerned.

EXPERIMENTAL

The transplantations, for the most part, were made on the basis of the results obtained with the monovalent sera. It was upon the theory of Loeb and Wright (1927) that the transplantations were largely made: that the presence of factors in the donor tissue which are absent in the host are responsible for tissue antagonism; that absence of factors in the donor tissue are of no consequence, assuming that the agglutinogens are an index of the factors responsible for the absorption of the foreign tissue. After it was discovered that a monovalent serum did not indicate incompatibility between the donor and host, the polyvalent serum was resorted to as a more sensitive test.

Most of the transplantations were made within 3 weeks of the test with the monovalent serum; tests with the polyvalent serum were made from 4 to 12 weeks after the transplantation experiments.

The grafts reported in these experiments involved most of the different integumental tissues, inasmuch as it has been previously demonstrated (Kozelka, 1931, 1932) that in the domestic fowl all the integumental characters persist equally well. The transplants were always grafted to contrasting regions of the bird; that is, wattle tissue was grafted to the shank or feathery portions of the body, scaly tissue to feathery integument, and feathery portions to scaly regions. Spurs, however, were always grafted to some part of the shank. The parts grafted always involved the entire thickness of the integument and approximated a square centimeter in size. Readings, with a few exceptions, were made weekly; the time of absorption indicated in the graft tables represents the first negative reading.

It must be borne in mind that there are all degrees of incompatibility between the donor tissue and the host. As a result of this variation, regression of tissues takes different forms. In the more extreme cases the graft was quickly absorbed or occasionally sloughed off. In other cases the process was slower—the grafted tissue became discolored or dry on the outer surface. This was usually the first indication of regression and became apparent either gradually or suddenly and at any stage of the graft. In still other cases the growth of the grafted structure simply appeared to be retarded. It is only reasonable to expect, in view of this variability, that it was not always easy to determine when the last vestige of foreign tissue disappeared, despite the fact that there was a contrast between the grafted portion and the surrounding tissue. Because of this difficulty, the time of absorption indicated in the graft tables must be regarded as approximate.

Strain 20. Agglutination Tables I and II; graft records, Table III.—The birds included under strain 20 were White Leghorns, representing three separate generations. Birds 578 and 625 were produced by back-crossing a male with his daughters. Birds 851, 852, 857, and 860 resulted from a mating of a half or full brother to 578 and 625; birds 883, 889, 893, and 913 were produced by mating females 578, 625, 851, 852, and 857 to a half or full brother of the last three mentioned birds.

TABLE I

AGGLUTINATION TESTS—STRAIN 20. MONOVALENT SERUM 1:15

Bird 1157 was a barred Plymouth Rock. In this and the following tables the signs have the following significance: +++, complete agglutination; ++, large clumps seen macroscopically; +, small clumps seen macroscopically; ±, clumps seen microscopically; tr., trace; f. tr., faint trace; −, no agglutination.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL										
	578	625	851	852	857	860	883	889	893	913	1157
578	−	tr.	−	−	+	−	+	+	+	±	−
625	++	−	++	++	++	++	−	−	++	−	−
851	−	±	−	−	+	−	+	+	+	+	tr.
852	−	f. tr.	−	−	±	−	+	±	+	±	f. tr.
857	++	+	++	++	−	++	−	−	−	+	+
860	−	−	−	−	±	−	+	±	+	±	−
883	++	+	++	++	+	++	−	−	+	+	±
889	++	+	++	++	++	++	−	−	++	+	±
893	++	+	+	++	−	++	−	−	−	+	tr.
1157	±	±	±	±	++	+	+	+	++	+	−

Agglutination tests with both the monovalent and polyvalent sera were made on all the birds except 860 and 893; these two birds were studied only with the monovalent serum. It will be observed from the agglutination Tables I and II that most of the birds in this strain fall into three fairly distinct groups; birds 578, 851, 852, and possibly 860 belong to one group; 883 and 889, to another; and 857 and 893, to still another group.

Of the transplants between individuals of similar groups, grafts 2, 6, 7, 13, 14, 15, 16, 17, 20, 24, 25, 26, 27, 28, 29, 32, and 33 were

TABLE II

AGGLUTINATION TESTS—STRAIN 20. POLYVALENT SERUM 1:15

The corpuscles of birds 625 and 34 were used in the development of the polyvalent serum. Bird 34, single-comb Brown Leghorn; bird 1197, rose-comb Brown Leghorn.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL									
	578	625	851	852	857	883	889	913	34	1197
578	−	±	−	−	+	+	+	±	+	+
625	+	−	+	+	+	−	−	−	±	tr.
851	−	+	−	−	+	+	+	tr.	+	+
857	++	+	+	++	−	−	−	tr.	−	tr.
883	++	+	++	++	+	−	−	±	+	+
889	+++	+	++	+++	+	−	−	tr.	+	±
913	+	−	+	+	+	−	−	−	+	±
34	+++	+++	+++	+++	+++	+++	+++	++	−	+
1197	++	++	+++	+++	++	+	+	+	±	−

TABLE III

GRAFT RECORDS FOR STRAIN 20

No. of Graft	Host	Donor	Type of Tissue	First Indication of Regression (in Weeks)	Weeks Persisted
1	578	578	Wattle	.....	14*
2	.....	851	Wattle	3	5
3	.....	851	Scaly	4	12
4	.....	851	Scaly	4	10
5	.....	852	Scaly	3	10
6	.....	852	Wattle	4	6
7	.....	852	Wattle	4	6
8	.....	860	Scaly	4	22†
9	.....	913	Scaly	.....	22†
10	625	889	Scaly	.....	22†
11	.....	913	Scaly	.....	22*
12	851	851	Wattle	.....	14*
13	.....	578	Scaly	2	4
14	.....	578	Wattle	3	5
15	.....	852	Scaly	2	4
16	.....	852	Scaly	2	5
17	.....	852	Wattle	4	5
18	.....	893	Ear lobe	4	6
19	.....	913	Scaly	−‡	.....
20	852	851	Scaly	4	4
21	.....	578	Scaly	4	9
22	.....	860	Scaly	−‡	.....
23	.....	889	Ear lobe	5	8
24	857	893	Scaly	2	5
25	.....	893	Ear lobe	2	4
26	860	852	Spur	2	3
27	.....	578	Spur	3	6
28	883	889	Scaly	4	5
29	.....	889	Wattle	3	5
30	889	625	Spur	3	4
31	.....	852	Spur	3	4
32	.....	883	Scaly	3	5
33	.....	883	Wattle	3	5
34	893	851	Spur	3	4
35	913	625	Spur	3	6
36	.....	578	Spur	7	7§
37	.....	851	Spur	6	6§

* Appeared normal throughout duration of experiment.

† Corneous stratum regressed slightly; scales barely evident.

‡ Negative.

§ Sloughed off.

absorbed within 6 weeks after transplantation; grafts 3, 4, 5, and 21 regressed more gradually; and only one graft (No. 8) of the twenty-two transplants persisted. In this particular case it will be noted from Table I that there is a slight indication of additional agglutinogens in the donor. While the scales were evident throughout the entire experiment, there was a slight indication of necrosis 4 weeks after the operation. There was also a general reduction in the size of the graft. It should be pointed out that in those cases where more than one transplantation was made from a single donor to a single host, the operations were performed several weeks apart. This probably accounts for the variability in the time the grafts persisted. That a difference in the physical condition of a bird is a factor of regression was suggested by the results obtained in previous work (Kozelka, 1931, 1932). But undoubtedly other factors also play a part in the regression of integumental grafts, inasmuch as it was observed that occasionally grafts made simultaneously from a single host showed a differential rate of absorption.

In all the remaining transplantations there was in each case a definite indication of additional agglutinogens in the donor of the grafted tissue. Grafts 9, 10, and 11 persisted; grafts 18, 23, 30, 31, 34, 35, 36, and 37 were absorbed. In grafts 9 and 10 there was a distinct difference between the donor and the recipient with respect to the agglutinogens; in the case of graft 11 the reactions of birds 625 and 913 were for the most part similar. The three grafts that did persist were reduced considerably in size. The reduction in size can, however, be attributed, at least in part, to structural, as well as to physiological, factors, as is occasionally the case with autotransplantations of similar tissues.

Strain 27. Agglutination Tables IV and V; graft records, Table VI.—The birds in this strain, as well as the birds included in strains 26, 25, and 24, were obtained from Dr. Knox of the Iowa State College of Agriculture as eggs with the numbers of both the hen and the cock indicated on each egg. All the birds in all four strains were originally derived from a single production cock, and two show hens of White Leghorns. The majority of the birds in the four strains had a coefficient of inbreeding slightly over 50 per cent; several, in which close inbreeding was employed only in more remote

TABLE IV

AGGLUTINATION TESTS—STRAINS 27 AND 24. MONOVALENT SERUM 1:15

The corpuscles of bird 34 were used in the development of the serum. Nos. 1133 and 1255, 1246 and 1247, 1252 and 1274, 1250 and 1251, 1277 and 1278, were full brothers and sisters, respectively. Bird 34 was a single-comb Brown Leghorn; bird 952, hybrid of a Brown and of a White Leghorn; bird 1368, barred Plymouth Rock.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL												
	Strain 27									Strain 24			
	1133	1162	1241	1246	1247	1252	1255	1256	1274	1250	1251	1277	1278
1133	−	−	−	−	−	±	−	−	−	±	tr.	−	−
1162	−	−	−	−	−	±	−	−	tr.	−	−	−	−
1241	−	−	−	−	−	−	−	−	−	−	−	−	−
1246	−	−	−	−	−	−	−	−	−	−	−	−	−
1247	tr.	tr.	±	−	−	+	±	±	±	±	±	±	±
1256	−	−	−	−	−	−	−	−	−	−	−	−	−
1277	−	−	−	−	−	−	−	−	−	−	−	−	−
1278	+	+	+	+	+	+	+	+	+	−	−	+	−
34	−	−	−	−	−	−	−	−	−	−	−	−	−
952	−	−	−	−	−	−	±	−	−	−	−	−	−
1368	+++	++	++	+++	++	−	++	++	++	−	−	++	++

TABLE V

AGGLUTINATION TESTS—STRAIN 27. POLYVALENT SERUM 1:50

The corpuscles of bird 1237 and two individuals belonging to this strain, though not included in the table, were used in the development of the polyvalent serum. For relationship of birds belonging to strain 27, see Table IV. Birds 1148, 1149, and 1237 were single-comb Brown Leghorns; bird 1157, barred Plymouth Rock.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL								
	Strain 27								
	1133	1162	1246	1247	1252	1255	1256	1148	1149
1133	−	−	−	−	+	±	±	tr.	±
1162	tr.	−	f. tr.	f. tr.	+	±	+	±	±
1246	−	−	−	−	+	tr.	tr.	±	±
1247	−	−	−	−	++	tr.	±	±	±
1252	++	++	++	++	−	+	++	−	−
1255	+	+	++	+	+	−	++	±	±
1256	−	−	−	−	±	−	−	−	−
1148	++	++	++	++	+	+	++	−	−
1149	++	++	+++	+++	±	+	++	−	−
1237	++	++	++	++	tr.	+	+	−	−
1157	++	++	++	++	tr.	+	+	−	−

generations, had only approximately 9 per cent. This degree of inbreeding was obtained by matings of half and full brothers and sisters together with sire-daughter matings.

All the transplantations in these four families were confined to individuals within a single strain. It is thus evident that in addition to the inbred stock used in these experiments, all the transplantations were made between individuals that were either half or full brothers and sisters.

TABLE VI

GRAFT RECORDS FOR STRAIN 27

No. of Graft	Host	Donor	Type of Tissue	First Indication of Regression (in Weeks)	Weeks Persisted	No. of Graft	Host	Donor	Type of Tissue	First Indication of Regression (in Weeks)	Weeks Persisted
38....	1133	1162	Wattle	2	6	49....	.....	1256	Wattle	3	5
39....	.....	1241	Spur	3	6	50....	1246	1133	Scaly	3	6
40....	.....	1241	Spur	3	7	51....	.....	1162	Wattle	3	4
41....	.....	1255	Spur	3	5	52....	.....	1241	Wattle	2	3
42....	.....	1255	Spur	3	5	53....	.....	1247	Spur	3	6
43....	1162	1133	Scaly	2	6	54....	1247	1246	Spur	3	4
44....	.....	1241	Scaly	2	4	55....	1252	1274	Scaly (2)	—*	.....
45....	.....	1256	Spurs (2)	3	4	56....	1255	1133	Spur	3	4
46....	1241	1133	Spur	3	7	57....	1256	1162	Spur	10	12
47....	.....	1162	Wattle	2	5	58....	.....	1241	Scaly	2	3
48....	.....	1246	Scaly	2	4						

* Negative.

Agglutination tests of all the birds used for grafting purposes in strain 27 were made with both the monovalent and polyvalent sera except birds 1241 and 1274; for these two birds tests with only the monovalent serum are available, owing to the death of the birds. While quite a number of birds in this group had practically identical reactions with either the monovalent or polyvalent serum, all the birds tested with both sera indicated more or less variation in their agglutinogens. Only birds 1241 and 1256 had identical agglutinogens; and tests for these, as already indicated above, were made only with the monovalent serum. Both of the reciprocal grafts (Nos. 49 and 58) were absorbed. All of the transplants between individuals in which there was no significant evidence of additional agglutinogens in the donor were likewise absorbed. In this group can be included grafts 46, 47, 48, 50, 53, and 57. It should, however, be observed that grafts 57 persisted longer than the usual period required for absorption. In all the remaining cases there was more or less evidence of the presence of additional agglutinogens in the donor. These also were all absorbed.

Strain 26. Agglutination Tables VII and VIII; graft records, Table IX.—In this group all except birds 1137 and 1260 are included in both tables; agglutination records involving these two birds will be found only in Table VII. While a number of birds had practically identical reactions with the monovalent serum, only birds 1253 and 1261 reacted similarly with both the monovalent and polyvalent sera. The one transplant between these two birds (No. 82) was absorbed within 4 weeks after transplantation. In the group in which no additional factors were evident in the donor, one (No. 85) persisted out of nine positive transplantations. In the absorbed group of this category are included grafts 63, 65, 70, 72, 77, 78, 81, and 83. In this group might also be included grafts 59 and 60. These undoubtedly were absorbed before the bandages were removed. On the basis of past experience it is not probable that both of the grafts would be negative in each of the two cases.

TABLE VII

AGGLUTINATION TESTS—STRAINS 26 AND 24. MONOVALENT SERUM 1:15

Nos. 1136, 1137, 1240, and 1280; 1242, 1243, and 1253; 1260 and 1261; 1250 and 1251, were full brothers and sisters, respectively

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL										
	Strain 26									Strain 24	
	1136	1137	1240	1242	1243	1253	1260	1261	1280	1250	1251
1136	−	−	−	−	−	−	−	−	−	−	−
1240	±	−	−	+	+	+	−	±	−	−	tr.
1242	−	−	−	−	−	−	−	−	−	±	−
1243	−	−	−	−	−	−	−	−	−	−	−
1253	−	−	−	−	−	−	−	−	−	−	−
1260	+	−	−	+	+	+	−	±	−	−	−
1280	±	−	−	+	±	+	−	tr.	−	−	−
1250	++	tr.	++	++	++	++	++	++	++	−	−
1251	++	±	++	++	++	++	++	++	++	−	−

Of the sixteen grafts in which there was evidence of additional factors in the donor tissue, graft 68 persisted; grafts 61, 62, 64, 66, 67, 69, 71, 73, 74, 75, 76, 79, 80, 84, and 86 were absorbed. In the cases of 75 and 76, there was only a slight indication of additional

TABLE VIII

AGGLUTINATION TESTS—STRAIN 26. POLYVALENT SERUM 1:15

Bird 1237, a single-comb Brown Leghorn, was one of the birds used in the development of the polyvalent serum; 1157, barred Plymouth Rock.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL								
	Strain 26								
	1136	1240	1242	1243	1253	1261	1280	1237	1157
1136	−	−	tr.	+	±	±	−	++	++
1240	+	−	+	++	+	+	−	++	++
1242	+	+	−	++	+	+	f. tr.	+++	++
1243	+	±	+	−	+	+	±	++	±
1261	−	−	−	±	−	−	−	+++	+
1280	++	++	+	++	+	+	−	++	++
1237	+++	++	+++	++	++	++	++	−	−
1157	+++	+++	+++	+	++	++	++	+	−

TABLE IX

GRAFT RECORDS FOR STRAIN 26

No. of Graft	Host	Donor	Type of Tissue	First Indication of Regression (in Weeks)	Weeks Persisted	No. of Graft	Host	Donor	Type of Tissue	First Indication of Regression (in Weeks)	Weeks Persisted
59	1136	1137	Spurs (2)	—*	.....	73	1243	1261	Scaly	5	7
60	.....	1240	Spurs (2)	—*	.....	74	.....	1280	Wattle	4	7
61	.....	1243	Scaly (2)	3	4	75	1253	1136	Scaly	3	6
62	.....	1253	Scaly	3	6	76	.....	1240	Scaly	3	4
63	.....	1260	Scaly (2)	3	4	77	.....	1242	Spur	4	6
64	.....	1261	Wattle	2	4	78	.....	1242	Spur	4	5
65	.....	1280	Spurs (2)	2	4	79	.....	1243	Spurs (2)	3	4
66	1137	1136	Spur	3	5	80	.....	1243	Wattle	3	5
67	1240	1136	Spur	3	5	81	.....	1260	Scaly	3	4
68	1242	1253	Spur	.....	24†	82	.....	1261	Wattle	3	4
69	1243	1136	Wattle	4	6	83	.....	1280	Scaly	3	6
70	.....	1137	Scaly	4	5	84	1260	1261	Spur	3	4
71	.....	1240	Scaly	5	8	85	1261	1260	Spur	.....	24†
72	.....	1260	Scaly	3	8	86	1280	1240	Scaly	2	3

* Negative.

† Appeared normal throughout duration of experiment.

agglutinogens; and these cases might have been included in the preceding category.

Strain 25. Agglutination Tables X and XI; graft records, Table XII.—All the birds in this strain except birds 1245 and 1258 showed more or less variation in the agglutinogens with either the monovalent or polyvalent serum. After the agglutination records were known, no transplantations were made between these two birds, owing to the emaciated condition of the individuals. Of the transplants between individuals in which there was only a slight or no indication of additional agglutinogens in the donor, all were absorbed within the usual time. In this group can be included grafts 92, 94, 95, 96, 100, and 101. In all the remaining transplantations there was a definite indication of additional agglutinogens in the donor. Grafts 87, 88, 89, 90, 91, 93, 97, 99, 102, 103, 104, and 105 were absorbed; grafts 98 and 106 persisted. Graft 98 was removed for histological purposes 12 weeks after transplantation; and in the case of graft 106, the host died 12 weeks after the operation. Both grafts would have undoubtedly persisted, inasmuch as they appeared perfectly normal at the time of the last observation.

TABLE X

AGGLUTINATION TESTS—STRAIN 25. MONOVALENT SERUM 1:15

Birds 1131, 1258, and 1259; 1138, 1139, 1244, and 1245, were full brothers and sisters respectively. Nos. 34, 1185, 981, 1150, and 1237 were single-comb Brown Leghorns; 952 and 959, hybrids of Brown and of White Leghorns; 1157 and 1368 barred Plymouth Rocks; and 1249, 1260, and 625, White Leghorns.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL						
	1131	1138	1139	1244	1245	1258	1259
34	tr.	+	+	++	+	+	+
1185	tr.	+	++	++	+	+	+
981	−	+	++	+	++	++	+
1150	++	+	++	++	++	++	++
1197	++	±	+	++	++	+	+
1237	++	±	+	++	++	+	+
952	−	−	−	−	−	−	f. tr.
959	−	f. tr.	±	−	tr.	±	tr.
1157	++	±	++	++	++	++	++
1368	+	±	++	++	+	+	+
1249	tr.	tr.	−	−	±	tr.	tr.
1260	++	++	++	+	++	++	+
625	++	±	++	+	++	+	++

TABLE XI

AGGLUTINATION TESTS—STRAINS 25 AND 24. POLYVALENT SERUM 1:15
Relationship of individuals is indicated in Tables IV and X.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL											
	Strain 25							Strain 24				
	1131	1138	1139	1244	1245	1258	1259	1251	1277	1278	1237	1157
1131	−	+	+	+	+	+	+	+	+	+	++	++
1138	−	−	+	tr.	+	+	±	−	+	f. tr.	+	+
1139	−	±	−	−	−	−	−	+	+	+	+++	++
1244	+	+	±	−	+	tr.	±	++	++	+	+++	++
1245	−	+	tr.	−	−	−	−	++	++	+	+++	++
1259	++	+++	+++	++	++	++	−	++	+++	++	+++	+++
1251	+++	++	+++	+++	+++	+++	++	−	++	+	++	f. tr.
1237	++	++	++	++	++	++	++	±	+	+	−	−
1157	++	+	++	++	+++	+++	+++	f. tr.	++	++	+	−

TABLE XII

GRAFT RECORDS FOR STRAINS 25, 24, AND 30

No. of Graft	Host	Donor	Type of Tissue	First Indication of Regression (in Wks.)	Wks. Persisted
Strain 25					
87....	1131	1138	Wattle	5	7
88....	.....	1139	Scaly	4	6
89....	.....	1244	Wattle	2	3
90....	.....	1245	Scaly	3	7
91....	.....	1258	Wattle	3	7
92....	1138	1131	Scaly	2	4
93....	.....	1259	Scaly	2	3
94....	1139	1131	Wattle	2	4
95....	.....	1244	Spurs (2)	3	7
96....	.....	1259	Wattle	2	5
97....	1244	1131	Scaly	4	6
98....	.....	1139	Scaly	.....	12*
99....	.....	1259	Scaly	3	6
100....	1245	1131	Wattle	5	6
101....	1258	1131	Scaly	2	3
102....	1259	1138	Wattle	4	6
103....	.....	1139	Scaly	4	7
104....	.....	1244	Wattle	4	7
105...	.....	1245	Wattle	4	8
106...	.....	1258	Wattle	.....	12†
Strain 24					
107...	1250	1251	Spur	.....	10‡
108...	1251	1250	Spurs (2)	3	5
109...	.....	1277	Scaly	2	3
110...	1277	1250	Wattle	.....	17§
111...	.....	1251	Wattle	.....	17§
112...	.....	1278	Wattle	.....	17§
113...	1278	1277	Scaly	2	3
Strain 30					
114...	888	887	Feathery	.....	16
115...	.....	922	Ear lobe	3	4
116...	922	887	Feathery	8	9
117...	.....	888	Feathery	3	4

* Normal. Removed for histological study 12 weeks after operation.

† Normal. Host died 12 weeks after operation.

‡ Normal. Host died 10 weeks after operation.

§ Characteristic wattle tissue at termination of experiment; reduced in size.

Strain 24. Agglutination Tables IV, VII, and XI; graft records, Table XII.—Only four individuals are included in this strain. Of the individuals used as donors, there was an indication of additional agglutinogens in all except bird 1278. In this particular case, bird 1277 apparently had all those possessed by bird 1278. The one transplant between these individuals (No. 112) with bird 1277 as the host persisted throughout the duration of the experiment, although there was indication of necrosis 4 weeks after the operation, with the result that the graft was somewhat reduced in size. In the six remaining grafts in which there was evidence of additional agglutinogens in the donor, though only slight in the cases of grafts 107 and 111, grafts 107, 110, and 111 persisted; grafts 108, 109, and 113 were absorbed. In the case of graft 107, the host died 10 weeks after the transplantation; but undoubtedly the graft would have persisted, inasmuch as there was no evidence of regression at the time of the last observation.

Strain 30. Agglutination Tables XIII and XIV; graft records, Table XII.—Strain 30 was originally obtained from a poultry farm in Pennsylvania as trap-nested eggs from a mating of six hens, supposedly all half sisters mated to a half brother. The following year one of the pedigreed males was mated to a half and to a full sister. The same two females, together with some of the daughters, were mated to an F_1 male the succeeding year. All of the individuals used for grafting purposes were from the latter mating.

It is evident from Tables XIII and XIV that there is a high degree of homozygosis as regards the agglutinogens between all the individuals of this strain, especially between birds 887 and 888; these two individuals have practically identical agglutinogens. Bird 922, on the other hand, has a fewer number, as far as is indicated by the monovalent serum. It may be significant that the results obtained with the monovalent serum were further supported by the results with the polyvalent serum with birds 895 and 922—the only two birds that survived until they could be tested with the polyvalent serum.

Of the two grafts with bird 888 as the host, the feathery tissue from bird 887 survived throughout the duration of the experiment, while the graft from bird 922 was absorbed within 4 weeks. However, the former graft never produced follicles, which condition was interpreted as a mild form of antagonism. This condition was previously encountered in a less-marked form where only feeble follicles were produced after long periods of time (see Kozelka, 1932). With

TABLE XIII

Agglutionation tests—strain 30. Monovalent serum 1:15

No. 840 was a half or full brother to the father of the remaining individuals.

Sera Absorbed with Cells of—	Corpuscles of Fowl					
	840	887	888	895	922	904
840	–	–	–	–	–	–
887	–	–	–	–	–	–
888	–	–	–	–	–	–
895	–	–	–	–	–	–
922	–	–	–	–	–	–
904	–	–	–	–	–	–

TABLE XIV

Agglutination tests—strain 30. Monovalent serum 1:15

The serum used in these tests was sensitized with the corpuscles of bird 34. Birds 1133, 1162, 1241, 1246, 1247, 1256, 1277, 1278, and 852 were single-comb White Leghorns belonging to strains 27, 24, and 20; Nos. 34 and 1187, single-comb Brown Leghorns; 952, hybrid of a Brown and a White Leghorn; 1368 and 1157, barred Plymouth Rock; and 1197 rose-comb Brown Leghorn.

Sera Absorbed with Cells of—	Corpuscles of Fowl			
	887	888	895	922
1133	–	–	–	–
1162	–	–	–	tr.
1241	–	–	–	–
1246	–	–	–	–
1247	–	–	–	–
1256	–	–	–	–
1277	–	–	–	–
1278	–	–	–	±
34	–	–	–	–
952	–	–	–	–
1368	+++	++	–	–
1157	++	++	–	–
852	–	–	–	±
1187	++	+	+	+
1197	++	+	–	–

bird 922 as the host, the graft from bird 887 persisted 9 weeks; the one from 888, 4 weeks.

Strains 31 and 32. Agglutination Tables XV and XVI; graft records, Table XVII.—The birds included in these two strains were produced by reciprocal crosses of strain 30 with single-comb Brown Leghorns of the same source. In strain 31 are included the offspring of two Brown Leghorn hens mated to a male from strain 30; in the reciprocal cross (strain 32), two females from strain 30 were mated to a Brown Leghorn male. The relationship where it was definitely known is indicated under Table XVI. The experimental work also included several pure-bred birds from parental strains. Agglutination Table XV includes only birds of strain 31 and one of the parental birds; Table XVI makes reference to all the living birds of this portion of the study.

TABLE XV

AGGLUTINATION TESTS—STRAIN 31. MONOVALENT SERUM 1:15

Birds 961, 962, 982, and 983, hybrid offspring of female 10; Nos. 963, 964, 1104, 1106, 1108, and 1112, hybrid offsprings of another female mated to the same male; 953, full brother to individuals of one of two groups; 887, White Leghorn from component family.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL												
	10	961	962	982	983	963	964	1104	1106	1108	1112	953	887
10	−	−	−	−	+	f. tr.	−	+	−	+	++	−	−
961	++	−	−	±	++	−	−	++	+	++	++	++	++
962	++	−	−	+	++	tr.	−	++	++	++	++	++	++
983	−	−	−	−	−	tr.	−	+	−	++	++	−	−
964	++	f. tr.	−	+	++	−	−	++	+	++	++	++	++
1108	tr.	−	f. tr.	f. tr.	+	−	−	−	−	−	−	−	−
1112	±	−	tr.	−	+	±	−	−	−	−	−	−	−
887	++	++	++	++	++	++	++	+	++	++	++	++	−

1. Transplantations between half or full brothers and sisters.—Twenty-one experiments are included in this phase of the study. It will be noted from agglutination Tables XV and XVI that birds 961 and 962 had practically identical reactions. The red blood cells of these two birds were tested with twenty-four additional absorbed, monovalent sera with a variation of not more than a single degree with any one serum. Of the reciprocal transplantations be-

tween the two individuals, the spur on bird 961 (graft 140) persisted and showed considerable development during the 14 weeks it was allowed to grow on the host, whereas both of the transplants (Nos. 144, 145) on bird 962 were absorbed. The two transplantations on bird 962 were made two months apart; the variation in the physical condition of the host might well account for the different rate of

TABLE XVI

AGGLUTINATION TESTS—STRAINS 31 AND 32. POLYVALENT SERUM 1:40

Birds 950, 959, 968, and 1113, hybrids from same mating as those described in preceding table; 952, 965, hybrids from reciprocal cross; 922, White Leghorn from component family; 1197, rose-comb Brown Leghorn; remaining individuals all of one general stock of single-comb Brown Leghorn.

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL											
	10	961	962	982	963	964	1104	1108	1112	1113	1114	1197
950	f. tr.	+	±	−	−	−	−	−	−	−	−	tr.
959	+	++	+	tr.	++	+	−	tr.	−	tr.	tr.	++
961	+	−	−	±	f. tr.	−	++	+	++	+	±	−
968	±	±	tr.	−	−	−	−	tr.	±	−	−	tr.
952	+	+	+	tr.	+	+	−	+	±	−	f. tr.	±
10	−	++	+	f. tr.	+	+	+	++	++	+	−	+
34	+	+	+	−	+	+	f. tr.	+	+	+	−	tr.
1170	f. tr.	+	+	−	+	+	tr.	±	+	+	−	tr.
910	+	++	+	±	tr.	±	++	++	+++	++	+	±
910 959	−	−	−	−	−	−	−	−	−	−	−	−
1197	+	±	±	tr.	+	±	+	+	++	+	±	−

TABLE XVI—*Continued*

SERA ABSORBED WITH CELLS OF—	CORPUSCLES OF FOWL												
	950	959	968	952	965	34	910	1170	1273	922	1370	1371	1372
950	−	f. tr.	−	+	−	±	±	+	±	−	f. tr.	f. tr.	−
959	+	−	+	+	+	±	++	+	++	++	±	±	±
961	++	+++	+	++	+	+	f. tr.	++	+	−	±	tr.	±
968	+	+	−	++	f. tr.	+	±	+	+	f. tr.	−	−	−
952	+	f. tr.	+	−	±	−	+	++	+	+	±	−	±
10	++	++	++	++	++	+	+	++	++	+	f. tr.	f. tr.	tr.
34	+	±	+	+	±	−	+	+	+	+	tr.	tr.	+
1170	+	±	+	+	tr.	−	f. tr.	−	±	+	−	−	f. tr.
910	++	+++	++	+++	+	+	−	++	+	+	+	+	+
910 959	−	−	−	−	−	−	−	−	−	−	−	−	−
1197	++	++	+	++	±	+	+	+	+	±	+	+	+

absorption. In those cases where there was only a slight or no indication of additional agglutinogens in the donor (grafts 126, 128, 146, 164, 168, 169, and 170), all were absorbed. It should be noted that grafts 128, 146, and 169 regressed gradually. In the category in which additional agglutinogens were evident in the host, grafts 142 and 171 each persisted 14 weeks, the time the experiments were terminated; grafts 137, 138, 147, 155, 156, 161, 162, 166, and 167 were absorbed. Of this number, grafts 147 and 161 persisted somewhat longer than the usual period required for absorption of grafted tissue. One of the grafts that was still evident at the end of 14 weeks (No. 171) showed indications of regression; the other appeared normal at the termination of the experiment.

In this group can be included the reciprocal grafts between the two individuals belonging to strain 32. Each individual had agglutinogens that were absent in the other. Graft 135 was absorbed within 4 weeks; while graft 149 was gradually absorbed from the third through the tenth week.

2. Transplantations between hybrids of reciprocal crosses.—Complete records are available for only two transplantations in this group. In the case where there were no additional agglutinogens (No. 127) the graft regressed gradually from the second to the fourteenth week; in the case of graft 148 in which additional agglutinogens were evident in the donor, the graft was absorbed in the usual time.

3. Pure-bred to hybrid.—In this series there are fifteen transplantations for which agglutination records are available. Not any of the birds can be considered to belong to a single blood group. In the three cases (grafts 125, 163, and 172) in which there was no significant indication of additional factors, all were absorbed in the usual time. In all the remaining transplantations there is more or less marked evidence of the presence of additional agglutinogens in the donor. All the grafts in this group were absorbed. Grafts 130, 133, 141, 151, 152, and 154 persisted 10 or more weeks; grafts 124, 132, 134, 143, 153, and 160 regressed within 7 weeks. It is of interest to note here that of the four transplants from mother to son, graft 141 persisted 14 weeks; grafts 143, 160, and 163 regressed within the usual period.

TABLE XVII

Graft records for strains 31, 32, and miscellaneous stock material

No. of Graft	Host	Donor	Type of Tissue	First Indication of Regression (in Weeks)	Weeks Persisted
118.....	10	961	Wattle	2	4
119.....	.....	962	Wattle	3	6
120.....	.....	982	Wattle	2	6
121.....	.....	983	Scaly	4	6
122.....	34	952	Scaly	2	3
123.....	910	968	Scaly	3	8
124.....	950	34	Scaly	2	7
125.....	.....	922	Wattle	3	4
126.....	.....	959	Wattle	2	3
127.....	.....	965	Wattle	2	14
128.....	.....	968	Wattle	3	12
129.....	.....	981	Scaly	2	6
130.....	.....	1170	Wattle	2	13
131.....	.....	1184	Wattle	5	13
132.....	.....	1273	Scaly	2	4
133.....	952	34	Wattle	2	10
134.....	.....	922	Wattle	2	4
135.....	.....	965	Scaly	2	4
136.....	.....	981	Scaly	3	5
137.....	959	950	Scaly	2	4
138.....	.....	968	Wattle	5	5*
139.....	.....	981	Wattle	2	11
140.....	961	962	Spur	.....	14†
141.....	.....	10	Wattle	2	14
142.....	.....	982	Wattle	6	14‡
143.....	962	10	Wattle	2	3
144.....	.....	961	Scaly	3	4
145.....	.....	961	Wattle	3	10
146.....	963	964	Scaly	3	12
147.....	964	963	Wattle	3	8
148.....	965	950	Scaly	2	5
149.....	.....	952	Wattle	3	11
150.....	965	981	Wattle	3	5
151.....	.....	1170	Wattle	2	13
152.....	.....	1273	Wattle	2	13
153.....	968	34	Wattle	2	5
154.....	.....	910	Wattle	2	10
155.....	.....	950	Scaly	2	5
156.....	.....	959	Scaly	2	7
157.....	.....	981	Scaly	2	7
158.....	.....	1184	Wattle	2	5
159.....	981	959	Scaly	2	....§
160.....	982	10	Wattle	3	6
161.....	.....	983	Scaly	3	9
162.....	983	961	Scaly	2	7
163.....	.....	10	Wattle	2	5
164.....	.....	982	Wattle	5	7
165.....	.....	982	Spur	5	....‖
166.....	1104	1108	Spur	2	5
167.....	.....	1108	Scaly	2	4
168.....	1108	1104	Spur	4	7
169.....	.....	1104	Wattle	3	9
170.....	.....	1112	Spur	2	6
171.....	1112	1108	Scaly	9	14**
172.....	1113	1114	Spur	2	6
173.....	1114	1113	Scaly	4	5
174.....	976	987	Scaly	2	...††
175.....	.....	984	Wattle	2	...‡‡
176.....	987	976	Scaly	2	3
177.....	.....	976	Wattle	2	5
178.....	1148	1149	Scaly	.....	14
179.....	1149	1148	Spur	.....	8§§
180.....	1369	1370	Spur	2	5
181.....	1370	1369	Spur	2	5
182.....	.....	1371	Spur	2	4
183.....	.....	1372	Spur	2	4
184.....	.....	1373	Spur	2	3
185.....	1371	1370	Spur	...‖‖	.....
186.....	1372	1370	Scaly	2	4
187.....	1373	1370	Scaly	2	4

* Sloughed off.

† Transplanted back to original donor; increased in length 3.5 mm. during the 14 weeks.

‡ Appeared normal at time of last observation.

§ Scales evident 8 weeks after operation, the time the bird died.

‖ Necrotic 7 weeks after transplantation, the time the bird died.

** Corneous stratum absorbed at time of last observation.

†† Scales evident 4 weeks after transplantation, the time the bird died.

‡‡ Scales slightly evident.

§§ Increased 3 mm. in length during the 8 weeks; transplanted back to original donor for another experiment.

‖‖ Negative.

4. From hybrid to pure-bred.—Seven transplantations are included in this series, four of which are from a son or daughter to the mother. All four of the transplants (Nos. 118, 119, 120, 121) from offspring to parent were absorbed within 6 weeks. In all except graft 120 there was a significant indication of additional agglutinogens in the donor. The three remaining grafts in this series, Nos. 122, 123, and 173, were absorbed in 3, 8, and 5 weeks, respectively. In each of the three cases there was evidence of additional agglutinogens in the donor.

5. Miscellaneous stock material.—One of the most interesting cases encountered in this study is that of birds 1148 and 1149 that were hatched from a shipment of eggs from a breeder in Indiana. As far as is known, the birds were not obtained from any inbred stock. It is, however, probable that the two birds were half or full brother and sister. Of the two reciprocal transplants (Nos. 178 and 179) between these two individuals, both persisted and appeared perfectly normal throughout the duration of the experiment. During the 8 weeks the spur was allowed to grow on the host, it increased 3 mm. in length. It will be noted from Table V that the two individuals had practically identical agglutinogens. It should be pointed out that the two individuals were not selected at random for the experiment but were selected from a number of birds on the basis of compatibility to reciprocal transplantations as infantile chicks.

In this series can also be included the transplantations between five birds that resulted from two generations of brother and sister matings. Agglutination records are available for only three of the positive grafts. While there was no significant indication of additional agglutinogens in any of the donors of the three grafts (Nos. 182, 183, and 186), all three were absorbed within 4 weeks after transplantation.

DISCUSSION

It is evident from the foregoing experiments that the factors responsible for the absorption of grafted tissue in the domestic fowl are independent of those causing agglutination with immune hemagglutinating serum. Of the positive grafts in the various strains, five were between individuals with similar hemagglutinogens, two from donors in which there was no evidence of additional agglutinogens, and eleven in which there was a more or less marked evidence of agglutinogens in the erythrocytes of the donor that were absent in those of the host. In the latter category there is an unquestionable presence of additional agglutinogens in the donor in seven of the eleven cases. The fact that the birds were, for the most part, inbred and that the transplantations were practically all made between half or full brothers and sisters would lead one to believe that the regression of grafted tissue is due to slight organic differences between donor and host—probably single protein differences. The rate of regression is undoubtedly directly proportional to the number of additional factors present in the donor tissue which are absent in the host.

The theory held by Loeb and Wright (1927), that incompatibility is due to the presence of factors in the grafted tissue that are absent in the host, is further supported by the reciprocal transplantations between pure-breds and hybrids. Though all the grafts from pure-breds to hybrids were absorbed, it will be noted that six of the fifteen persisted 10 or more weeks. This can be considered significant in the light of the small number of grafts that persisted between birds of inbred families and the time of regression of those that were absorbed. Of the seven grafts from hybrids to pure-breds, six were absorbed within 6 weeks; only one persisted 8 weeks. This would seem to indicate that the larger number of factors expected to be found in a hybrid host offers a greater opportunity for compatibility between the transplant and the host.

How the small percentage of grafts that persisted in these experiments is to be reconciled with the results obtained with infantile chicks is difficult to explain. Considering all the transplants between the individuals presented in these experiments, the large majority were between half or full brothers and sisters of inbred strains, only 10.2 per cent persisted. In the work with infantile chicks 18 per cent of the grafts between unrelated birds persisted, 27 per cent between a population consisting of half or full brother and sister, 50 per cent between full brother and sister, and 68 per cent between offspring from father-daughter matings (Kozelka, 1931, 1932). Technique cannot be considered a factor, inasmuch as the same method was employed. Furthermore, the grafts were examined especially for attachment to the host at the time the bandages were removed, and all doubtful cases were considered negative. We note here, too, that of a number of autoplastic grafts not reported elsewhere, some of which were spurs 2–5 mm. in length, all were positive. Undoubtedly the adult has greater resistance to foreign tissue than the chick, but then one would expect that the grafted tissue would regress upon the development of the factors of resistance as the chick grows older unless there is a greater ability in the young tissue to adjust itself to the infantile host in those cases where the biologic differences are only slight. There is some evidence in support of this view even in transplantations between adults. A number of grafts that are listed as positive did show signs of regression in certain stages of the graft and yet maintained most of their outer characteristics. In some cases regression was more marked; the scaly stratum appeared to "melt" to the extent that the scales were barely visible, and remained in this condition throughout the experiment. The writer is inclined to the view that in the milder forms of tissue antagonism the host is capable of eliminating the incompatible elements without totally destroying the tissue. This is further supported by work previously reported (Kozelka, 1929, 1932) on the transplantation of the spur. In those cases where the spur normally developed, occasionally cases were encountered where the spur remained infantile or developed only slightly.[3] Unless one assumes that adult tissue has less power to adjust itself to the adult host than young tissue to an infantile host, the difference in the percentage of grafts persisting between infantile chicks and adults cannot be explained.

Whether these results can be applied to transplantations between human beings may still be disputed. But on the basis of the results obtained by Landsteiner and Levine (1928*a*) with anti-human erythrocyte serum, the complexity of the human blood is not unlike that of the fowl. By the use of a technique similar to the one employed in these experiments it was possible to distinguish thirty-six subgroups disregarding intensity of reaction in the blood of human beings, and even a greater complexity was postulated. In view of the similarity of the blood in the two species of animals with practically an identical technique, and the fact that negative results were obtained in the fowl, it seems highly improbable that the principle underlying incompatibility in humâns should be as simple as the one based on human iso-agglutinins. Besides, iso-agglutinins exist in the fowl as well as in the human being (Landsteiner and Miller, 1924). While no attempt has been made to correlate these with tissue antagonism, the occasional reaction obtained cannot be interpreted as indicative of incompatibility in view of the small percentage of grafts persisted. The fact that only an occasional mild reaction is obtained with iso-agglutinins in the fowl and that it is regularly obtained in humans indicates, if anything, that a greater homogeneity exists with respect to the agglutinogens in the fowl than in humans.

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[3] It is definitely known that incompatibility would not account for all the exceptions.

CONCLUSIONS

Transplants between individuals with practically identical or no additional agglutinogens, demonstrable by the technique employed, in the donor are absorbed about as frequently as transplants between individuals in which there are agglutinogens in the donor that are absent in the host. This can only mean that the factors responsible for the absorption of foreign tissue are not identical or associated with the factors causing agglutination with immune hemagglutinating serum. The fact that only a small percentage of the homoioplastic grafts between half and full brothers and sisters in inbred adult birds persisted points to the conclusion that the regression of grafts is due to slight biologic differences; the rate of regression is undoubtedly determined by the extent of differences between donor and host.

In view of the similarity in the complexity of both chicken and human blood as indicated by immune serum, it does not appear probable that iso-agglutinins in humans are indicative of incompatibility in tissue transplantations.

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REGENERATION IN PHAGOCATA GRACILIS (LEIDY)

(Three figures)

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IN THE large body of literature dealing with the physiology and regeneration of flatworms, *Phagocata gracilis* has been mentioned in comparatively few instances. T. Morgan (1900) included *Phagocata* with other flatworms in a study of regeneration at surfaces cut at various angles with the long axis of the animals. F. R. Lillie (1902), in a brief paper on regeneration in *Planaria,* described regeneration in this form with special reference to the size of the smallest part capable of reconstitution. Morgan and Schiedt (1904) studied the regeneration of the pharynges. L. Morgan (1906) published the results of an extensive series of grafting experiments with *Phagocata.* Walter (1907) studied the reaction of the animal to light. Child (1915, p. 101) states that young animals are more susceptible to toxic agents than are older animals. Curtis and his students (Curtis and Schulze, 1924; Isely, 1925) have reported brief notes on the rôle of formative cells in the regeneration of *Phagocata.* A study of the location of the mouth in regenerates derived from different regions of the animal was published by the present writer (Buchanan, 1927). I have been unable to find any other references to its physiology or regenerative characteristics. The descriptive literature is also meager, Woodworth's paper (1891) and that of Peasley (1910) being the only fairly complete studies of the morphology of *Phagocata gracilis.*

This paper is a study of the regenerative capacity of *Phagocata* as indicated by differences in the regulation of body form in regenerating transverse pieces. In addition to a contribution to the knowledge of the characteristics of the animal, some facts appear that have a bearing on the general problem of regeneration.

MATERIALS AND METHODS

Phagocata are easily obtained in large numbers from spring-fed pools and streams in and about New Haven. Ordinarily, stocks were secured in late autumn for work during the winter months and were discarded for fresh collections with the coming of summer. The animals were kept in large quantities of tap water and fed twice each week with beef liver. With proper care and in the absence of infection the animals thrived; unless the food supply was regulated they grew to be much larger than any that appeared in fresh collections. In late winter egg capsules were deposited in the pans. Only healthy and apparently normal animals were used for experimental purposes. The data given in the present communication are taken from observations and experiments extending over a period of about 7 years, during which time thousands of animals were handled.[1]

The details of the operation of cutting and the care of regenerates were so simple that they need not be described. While data obtained from a variety of different types of experiments are at hand, attention is confined here to the behavior of transverse pieces, with special reference to the form, amount of new tissue developed, and time required for the reconstitution of a new individual from pieces with anterior ends taken from the same region of the animal but representing different fractions of the whole, and from pieces representing the same fraction but taken from different regions. The levels of section and size of fractions are shown in Figure 1.

In determining the extent of new tissue developed in reconstitution, measurements were made 3 weeks after section. At this time the regenerates move about freely, feed, and in general have the form of animals of corresponding length taken from the natural habitat, except that the position of the mouth has not yet been established at the normal level. Measuring was accomplished by first anesthetizing with chloretone until the animals were entirely inert. They were then individually placed on a stage micrometer and spread out, the excess liquid being drawn off with a pipette. Tissue newly formed at the cut surfaces remains free from pigment for

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[1] The work was done in the Osborn Laboratories, Yale University. Acknowledgment is made for the facilities and materials provided.

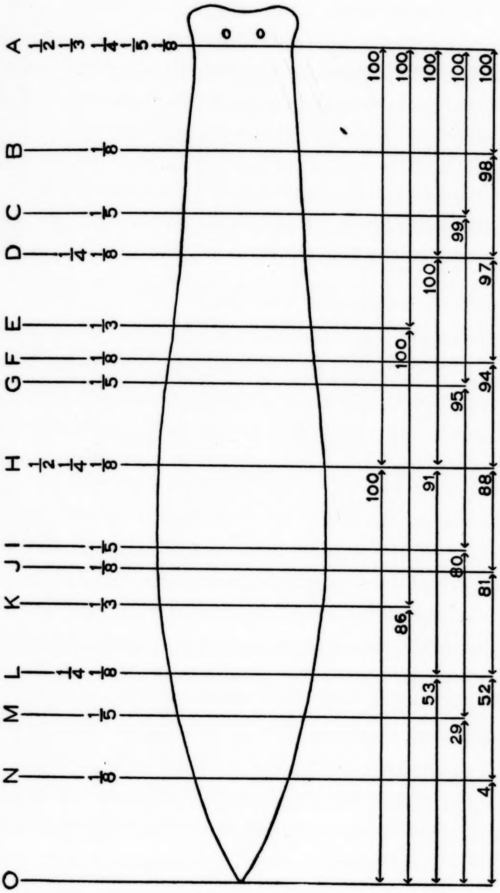

FIG. 1.—Scheme showing the percentage of cross-pieces of *Phagocata gracilis* derived from different regions and representing different fractions of the whole animal that regenerate complete individuals. The percentages are derived from the results obtained in the regeneration of not less than two hundred pieces from all the regions and all the fractions indicated. In all, more than fifteen hundred animals were cut, ranging in length from 11 to 14 mm. The letters indicate the levels of section; the fractions correspond to approximate fractions of the whole animal exclusive of the head anterior to the level A; the percentage of regeneration is shown for each fraction on the corresponding lines below the outlined animal.

several weeks; so it was not particularly difficult to distinguish a line between the newly formed structures and the old tissues of the piece. The reliability of this method of measurement has been shown in some detail elsewhere (Buchanan, 1927). Relatively small standard deviations indicate that the individual variations are within narrow limits and that the data so obtained are substantially accurate. It was found convenient to measure twenty animals in each experiment; consequently the data represent the average outgrowth of anterior and posterior tissue in twenty regenerates in each lot approximately 21 days after the pieces were cut.

FORM OF REGENERATED HEADS

A transverse piece from any region of the animal either develops a head that is normal in every respect or else does not form any sort of head, the wound being merely drawn together and filled with scar tissue. Under the binocular microscope the first visible indication of regeneration is the formation of a white film over the anterior cut surfaces. As will be noted later, the time required for the first appearance of this film varies with the level of origin of the piece. The central region of the film becomes more conspicuous and presently appears as a rounded knob of pigment-free tissue. The cephalic lobes now make their appearance, being first indicated by the change in shape of the rounded blastema to a more angular form. Eyes are detectable at this time, at first as white, semitransparent spots but very soon acquiring the black pigment of the normal eye. The eyes are always two in number and always normal in position and shape. In pieces taken from the anterior region of the intact animal the outgrowth of new tissue that forms the head is distinctly less and appreciably shorter than in pieces representing the same fraction of the animal but taken from more posterior levels.

In the case of pieces that do not develop heads, anterior regeneration is limited to the formation of a wedge-shaped mass of pigment-free tissue filling the notch formed as the lateral margins of the piece are drawn medially. Headless pieces resulting from sections with both anterior and posterior cut surfaces are relatively few in number. Posterior regeneration in such cases is limited to the healing of the wound; so far as observations with the binocular go, except as noted below, such pieces undergo no regulation toward the normal form.

Headless pieces may be kept alive without food for at least 5 months. In headless types formed by pieces taken from the extreme posterior end so as to include approximately the most posterior one-eighth of the animal, no changes in form occur, except that the piece becomes conical in shape. In headless forms that originate from pieces representing approximately the most posterior one-fourth of the animal, certain changes may occur long after the wound has filled with scar tissue. After 2 or 3 months it is noted that in a few cases a more or less complete regulation toward the normal is being effected. The apex of the cone formed by the lifting up of the old tissue owing to the contraction of the margins of the piece becomes bluntly pointed and inclines forward. It comes to overhang the scar of the healed wound, which assumes the position of a shelf of tissue reflected ventrally under the conical point (Fig. 2). The motility of the piece increases, and the inclined cone behaves more or less like a head. In rare instances (not more than four of these cases have been observed) eyes develop, and the piece eventually assumes the form of a normal animal. The new head arises from the region dorsal

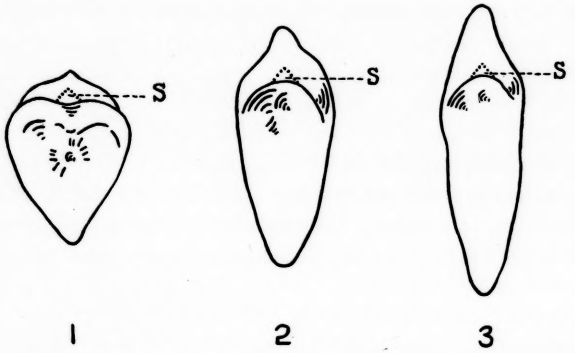

FIG. 2.—Three stages indicating the changes in headless one-quarter pieces derived from the most posterior one-quarter of the whole animal. Ventral surfaces. Redrawn from free-hand sketches made during the fourth and fifth month after isolation. *S* indicates the position of the scar tissue.

and slightly posterior to what had been the original anterior cut surface.

TIME INTERVALS BETWEEN ISOLATION AND RE-DIFFERENTIATION

Much evidence, including that of Schmalhausen (1930), has shown that in the development of the organism growth decreases as differentiation becomes more and more complete. Degree of differentiation may therefore be employed as a criterion of completeness of reconstitution in the regeneration of pieces of *Phagocata*. To determine the differences in time requirement for regulation in pieces from different regions, I selected the first appearance of the blastema and the first visibility of the eyespots as end-points or criteria of progress. In the most extensive experiment, seventy-five animals approximately 14 mm. in length were selected and divided into groups of twenty-five. The heads were cut off just posterior to the eyes. Animals in the first group were cut into approximate anterior and posterior halves, all anterior pieces being placed in one container and all posterior pieces in another. The members of the second group were cut into approximate fourths, all pieces from the same region being placed in the same container, as before. Similarly, the animals in the third group were cut into eighths and the pieces from the various levels segregated. Each day following all pieces were examined under a low-power binocular, and the extent of anterior and posterior regeneration recorded. Particular attention was paid to the first appearance of eyespots in members of each group, since their presence may be taken to indicate approximately the same degree of regulation in all pieces regardless of size or origin.

As shown in Table I, eyespots appeared on the sixth day in pieces taken from just behind the head (A pieces, Fig. 1), regardless of the size of the fraction that composed the original piece. It was quite clear that the time required for eyespot differentiation is independent of the amount of tissue in the piece, within the limits employed. But more time is required for eye formation as the level of the piece becomes more posterior. For example, pieces taken to include the level H (Fig. 1) representing different fractions of the whole animal but containing the same region as their anterior ends (HO $\frac{1}{2}$, HL $\frac{1}{4}$, and HJ $\frac{1}{8}$ pieces) require more time than do A pieces, but the time interval is independent of the size of the fraction. Regenerates derived from other regions also show these same characteristics; the time required for eye differentiation increases as the level of section becomes more posterior, but is independent of the size of the piece.

The increase in time required for differentiation as the level or origin becomes more posterior is also indicated by some supplementary observations on the righting reaction of regenerated individuals. If regenerates derived from the anterior region of the animal are turned ventral surface upward, they promptly right themselves. But when regenerates derived from posterior regions are similarly treated, their response is distinctly sluggish and the righting is accomplished only after several attempts. Whether this difference is due to differences in the functioning of muscles or of nerves is not essential; in either event it represents a difference in extent of acquirement of normal behavior.

TABLE I

TIME OF APPEARANCE OF ANTERIOR AND POSTERIOR TISSUE AND OF EYESPOTS

Pieces designated as in Figure 1.

Fraction	Anterior Regeneration (Days)	Posterior Regeneration (Days)	Eyes (Days)
AH ½	1	4	6
HO ½	2	....	10
AD ¼	1	3	6
DH ¼	2	4	8
HL ¼	2	3	8
LO ¼	3	....	12
AB ⅛	1	2	6
BD ⅛	2	2	6
DF ⅛	2	2	8
FH ⅛	2	2	10
HJ ⅛	2	3	10
JL ⅛	3	5	12
LN ⅛	3	5	15
NO ⅛	Scar tissue visible on third day No regeneration		

Notations were made concerning the rapidity of growth of new tissue in pieces from different regions. The blastemas become visible first in pieces from the anterior region of the animal, as is shown in

Table I. Moreover, in any piece the head blastema is first to form. During the early period of regeneration new tissue forms more rapidly in anterior pieces and more rapidly at the anterior than at the posterior end of a piece.

These results are in general accord with those of others. Thus Morgulis (1909) found that the rate of regeneration of pieces of the polychaete annelid, *Podarke obscura*, decreases as the level of section becomes more posterior. Child (1911) found the same to hold true within the zooid for pieces of *Planaria dorotocephala*, and Abeloos (1927) showed similar facts concerning the regeneration of pieces of *Planaria gonocephala*. Coe (1929) states that the rate of regeneration of pieces of the nemertean, *Lineus socialis*, decreases as the region from which the piece is taken becomes more posterior. It may be pointed out, however, that the term "rate," as frequently used, is inferred from differences in time of completion of regulation. It must be considered subject to the reservation that the differences observed may in part be due to increasing delay in the initiation of the processes of regeneration as the level of section becomes more posterior, not necessarily solely to decreasing rates of growth and of differentiation. It seems probable that the differences in rate of growth observed during the early stages of regeneration of pieces of *Phagocata* disappear or are even reversed later, since at the end of 3 weeks the total length of new tissue formed is very nearly the same in pieces from all regions (see Table II).

LENGTH OF NEW TISSUE REGENERATED ANTERIORLY AND POSTERIORLY

Preliminary observations indicated that the extent of new tissue developed in the process of regulation was different as between the anterior and posterior ends of an isolated piece, and differed in pieces from different regions of the whole animal. A three-dimensional measurement in order to determine the volume of new tissue was not feasible. Consequently I determined the length of new tissue at the head and tail ends of the regenerates and employed that single dimension to represent the relative volumes. This is open to objections, for head and tail are not the same shape. However, an inspection of Figure 3 shows that length may serve quite satisfactorily as a rough index of the quantity of new, unpigmented material formed in the regulatory process.

When regenerates derived from pieces representing approximately equal fractions of the original animals are measured the third week after isolation, it is found that the average length of the animals is very nearly the same regardless of the region of origin of the pieces (Table II). Moreover, the data show that the length of the regenerate is not directly proportional to the fraction taken; one-half pieces do not form individuals that are twice the length of one-fourth pieces, for instance. Furthermore, the smaller the fraction taken, the greater the proportion of total new tissue formed in the regulatory process.

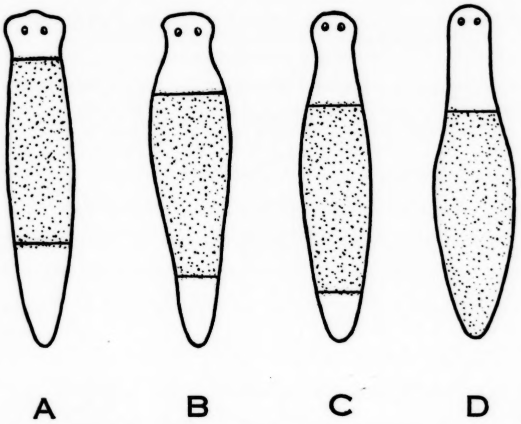

FIG. 3.—Position and extent of new and old tissue in one-quarter pieces measured 3 weeks after isolation. *A*, the most anterior fourth; *B*, the second fourth; *C*, the third fourth; *D*, the most posterior fourth.

The more posterior the region of origin of the piece the longer the new anterior, and the shorter the new posterior tissue. This is shown in Table II and in Figure 3. Thus the old tissue forms a more anterior region of the regenerate in those derived from the anterior than it does in regenerates derived from more posterior regions. These facts hold true regardless of the size of the fraction that comprises the original piece.

POTENTIAL FOR COMPLETE REGENERATION

The data on the percentage of heads formed in mass experiments are represented by a scheme in Figure 1. For convenience the percentage of heads in pieces from a given level or of a given size will be referred to as the "head frequency." Since complete individuals are formed by all pieces that regenerate heads and since no appreciable regulation occurs in the absence of head formation, this term may also be taken to represent the percentage of pieces that complete regeneration under the conditions of the several types of experiments.

TABLE II

TOTAL LENGTH, LENGTH OF ANTERIOR AND POSTERIOR NEW TISSUE, RATIO OF ANTERIOR TO POSTERIOR NEW TISSUE, AND RATIO OF NEW TO OLD TISSUE

Pieces cut from 15 mm. animals and measured after 3 weeks' regeneration. Each figure represents the average of twenty regenerates. Level of section as in Figure 1.

FRACTION	LENGTH (MM.)				RATIO		
	Total	Av.	Ant.	Post.	A/P	New/Old	Av.
½ AH	7.02	6.84	0.76	1.23	0.61	0.28	0.28
HO	6.66		1.33	....	....		
¼ AD	4.74	4.63	0.63	1.49	0.41	0.44	0.45
DH	4.83		1.20	1.10	1.09	0.47	
HL	4.75		1.41	0.66	2.13	0.43	
LO	4.22		1.37	....	....	......	
⅛ AB	3.69	3.57	0.54	1.37	0.39	0.51	0.57
BD	3.54		0.86	1.24	0.69	0.59	
DF	3.66		0.98	1.08	0.90	0.56	
FH	3.43		1.07	0.92	1.18	0.57	
HJ	3.50		1.24	0.85	1.45	0.59	
JL	3.64		1.42	0.73	1.94	0.59	
LN	3.62		1.54	0.55	2.80	0.57	
NO	Only one in this group regenerated						

Head frequency in relation to size of fraction; pieces cut with anterior ends from the same level.—From the scheme in Figure 1 it is clear that in pieces cut so as to include tissues just posterior to the head as their anterior ends, the head frequency is not related to the size of the fraction or to the amount of old tissue taken. Pieces from this region form heads in 100 per cent of the cases regardless of the size, within the limits employed here. But in regenerates derived from more posterior regions, cut so that the anterior end is made up of more posterior material, the size of the fraction has some relation to head frequency. For example, at the level of the mid-region, H in the scheme in Figure 1, if the anterior end is a part of posterior half-pieces, the head frequency is 100 per cent; if the same region is the anterior end of a one-fourth piece, the head frequency is 91 per cent; if it forms the anterior end of a one-eighth piece, the head frequency is 88 per cent. Furthermore, when one-fifth pieces are cut to include the level I as their anterior ends, the head frequency is 80 per cent; but in one-third pieces cut to include the more posterior level K as the anterior end, the head frequency is appreciably greater, 86 per cent. These facts make it clear that the nearer the posterior cut surface of the piece is to the anterior, the lower the head expectancy. But this relation between the size of the fraction taken from posterior regions and the head frequency is not intimate; differences in quantity of tissue forming one-half, one-fourth, and one-eighth pieces are not reflected as corresponding differences in head frequency. The data record similar facts as regards all regions posterior to the level A.

Head frequency in pieces of the same size when the pieces are taken from different regions.—The data in Figure 1 are taken to show that, as pieces representing approximately the same fraction of the whole animal are taken from more and more posterior regions, the head frequency decreases. Particularly is this true of pieces taken from regions posterior to the level H. For example, the head frequency of one-eighth pieces taken to include the level H as their anterior end is 88 per cent; when taken to include the level L as the anterior end, it is 52 per cent, and at the level N, 4 per cent.

When the data on head frequency are examined, it is seen that two conditions tend to reduce the probability of head regeneration in any given piece: (1) some property of the tissues of the intact animal that decreases head frequency as the level from which the piece is taken becomes more posterior, and (2) the approximation of the posterior to the anterior cut surface of the piece. The latter influence is not apparent when pieces are taken from just posterior to the head; in no region does it inhibit head regeneration very markedly. For example, at the level L the head frequency of one-fourth pieces, which have no posterior cut surface is only slightly greater than that of one-eighth pieces, in which the posterior cut is very near the anterior.

The unavoidable inaccuracies of free-hand cutting of pieces afford an opportunity to study the behavior of the same region of the intact animal when cut to form the anterior or the posterior end of a piece. Thus, when pieces are cut as halves, some contain the level H as their posterior ends, others have the same relative region of the animal as their most anterior tissues. In the former case the tissues regenerate a tail and show the characteristics of posterior regeneration described above. In the latter case they exhibit the properties of head-forming tissues.

DISCUSSION

A number of investigators have observed and described the aggregation of certain types of cells at cut surfaces of regenerating pieces of planarians, nemerteans, and annelids, and have described at least some of the histological events accompanying the formation of the blastema (e.g., Nusbaum and Oxner, 1910; Krecker, 1923; Coe, 1929; Stone, 1930; Faulkner, 1932). Emphasis upon the presence of neoblast or formative cells and nerve tissue implies that regeneration depends upon the presence of specific tissues. But the principles that control tissue development cannot be ignored. In attempting to account for the facts of regeneration in *Phagocata gracilis* in terms of the quantity of tissue present in the piece—e.g., neoblasts or nerve tissue—insurmountable difficulties are encountered, for it seems necessary to assume that head-forming cells migrate solely anteriorly and tail-forming cells posteriorly. If this line of reasoning is followed, one arrives at the untenable conclusion that neoblast cells are self-directive, that the structure to be regenerated is in effect pre-formed in the intact animal, and that when a piece is isolated the pre-determined elements are oriented by self-contained factors. This brings one dangerously near to endowing neoblast cells or nerve tissue with entelechies. It cannot be denied that the blastema is derived from certain types of tissue, nor that the presence of nerve is necessary in some forms at least. However, the results reported here, if considered alone, show that the sort of blastema formed, the time required to form it, and its developmental fate depend on its position with reference to the piece as a whole and are not inherent properties of any special tissue. It is unthinkable that a given cell or group of cells regularly develop a head when at the anterior end of a piece and regularly develop a tail when at the posterior end, without being influenced and controlled by factors outside themselves.

Other and specific objections to interpreting the facts in terms of properties inherent in specific types of cells may be mentioned. For example, the inhibitory effect on head formation is much greater if the level of origin of the piece is shifted posteriorly than if the size of the piece is reduced. Presumably, in reducing the size of the fraction from one-half to one-eighth the total length of the animal, one reduces the number of neoblasts and the quantity of nerve tissue correspondingly; yet the reduction in head frequency is small as compared with the effect of shifting the level of origin of the piece.

Furthermore, the data in Table II are interpreted to mean that the proportion of new to old tissue formed during regeneration is much the same at all levels and that this ratio increases as the size of the fraction of the whole diminishes. Thus, it seems impossible to consider the formation of new tissue as an expression of the number of formative or neoblast cells, since these, according to Curtis and Schulze (1924), decrease in number from anterior to posterior. Nor do these facts support the thesis so long defended by Loeb that regeneration may be referred to a Law of Mass Action. For in pieces of *Phagocata*, the smaller the fraction taken the relatively greater the quantity of new tissue formed. This is just the opposite of expectation according to Loeb's theory of regeneration. Differences in head frequency brought about by moving the level of origin of the pieces posteriorly and by cutting them smaller must be referred to some property of the tissue that decreases from anterior to posterior or is affected by the act of cutting; differences in quantity of any specific tissues are eliminated.

While pieces that are capable of regenerating a new head are equipotent regardless of the region from which they are taken, the data show clearly that the conditions that initiate and influence regulation are by no means identical in all pieces. Thus, if a piece is taken from the region just posterior to the old head, the time required for head formation is less, the new anterior tissue is less in quantity, and the regenerate assumes the normal reactions sooner than do regenerates from more posterior regions. Moreover, the potential as regards head or tail regeneration depends upon whether the tissue forms the anterior or posterior region of the piece, not upon the position of the tissue in the original animal. For example, at the level H, Figure 1, the tissue forms the tail if it is attached to an anterior half, or forms a head if a part of a posterior half. Furthermore, if a given region is forming a head, the regenerative processes are initiated and the normal structure is formed sooner than if the same mass of tissue is forming a tail. Clearly, the determination of the regenerative value of any given region is associated with the polarization of the piece which includes it.

The facts at hand are sufficient to indicate the nature of the properties which determine the type of structure that is realized at the cut surfaces of a regenerating portion. Since a piece, when the regenerative processes are initiated, is still composed of structures and tissues that are essentially parts of the original intact animal, such tissues may be expected to continue to function as adult structures for a longer or shorter period following the isolation of the piece. The factors that determine the regeneration of a new individual must be in some way associated with the functioning of the tissues as they compose the intact animal and with the functional changes resulting from isolation. The inquiry into the nature of these factors begins, therefore, with an examination of the physiological properties that are not identical in different regions of the intact animal.

The data show quite distinctly that pieces from anterior regions differentiate a new head in less time than do pieces from more posterior regions. They show, further, that anterior pieces are first to regain the co-ordination necessary for the righting reaction. These facts can only be interpreted to mean that the type of metabolism that is concerned in differentiation is most active in pieces from anterior regions and is less and less active as the level from which the piece is taken becomes more and more posterior. Whether or not an antero-posterior gradient in rate of oxidative metabolism can be demonstrated in the adult intact animal has no important bearing on the problem under discussion. The data showing differences in rapidity of attainment of differentiation are not open to serious doubt, whereas differences in functional activity of integumentary glands, of the digestive system, and other temporary functional conditions may more or less completely mask a gradient in rate of oxidative metabolism, if one exists.

A distinction between metabolic processes concerned in differentiation and those concerned in growth is commonly recognized; from various lines of evidence it is clear that the two phases of development are accurately synchronized in the production of normal form. In pieces of *Phagocata* growth begins first in sections taken from the anterior region and first at the anterior end of each piece. Thus, differences in the time of appearance of new tissue at cut surfaces and differences in rate of growth of new tissue in the early period of regeneration show that the metabolic processes concerned are slower and slower as the level from which the piece is taken becomes more posterior. It is evident from the facts at hand that, as regards rate of growth during the early stage of regeneration of a new individual from a fragment and growth after the organization is effected, there is a marked difference. For it was clearly shown in previous work (Buchanan, 1927) that in animals freshly collected and in animals that had completed regulation growth is more rapid posterior to the mouth than elsewhere along the long axis of the animal. If the interpretation of the data is correct that both growth and differentiative metabolism are slower in freshly isolated pieces as the level of origin becomes more posterior, and no other interpretation appears possible, then in any isolated piece the tissues adjacent to the anterior cut surface are most active in those phases of metabolism that are concerned in reconstitution. Moreover, the conclusion cannot be avoided that such active tissues are the leading elements in the process of regulation, for the head always forms first and forms from new tissues produced at the anterior cut surface. Furthermore, it appears that this relatively greater activity of the tissues adjacent to the anterior cut surface must have a certain threshold value in order that a head be formed, for pieces either regenerate complete normal heads or no heads at all. The relatively slower metabolic processes in the anterior tissues of pieces as one takes sections from more and more posterior regions seems adequate to account in part for corresponding decreases in head frequency shown in the results of mass experiments.

It was shown that the shorter the piece with anterior end taken from a given region, the less frequent the regeneration of a head (except in pieces with anterior ends taken just posterior to the eyes). Obviously, the approximation of the posterior cut surface to those tissues that are most active in regenerative metabolism in some way inhibits the regulatory processes. For the nature of this inhibitory action it is necessary to examine the physiological effects of injury. Most conspicuous among these effects is the increase in metabolic activity of various sorts, not only in the injured tissue but in regions more or less distant. Unpublished data at hand show that the oxygen consumption, carbon dioxide output, and susceptibility to poisons of freshly cut pieces of *Phagocata* are greater than in the intact animal. Moreover, the fact that growth and differentiation follow injury constitutes prima facie evidence that the metabolic processes concerned are affected by injury; substantial direct evidence is also found in the fact that short pieces develop fewer heads than do long pieces.

Since moving the level of origin of the pieces posteriorly and cutting the piece shorter are additive in their effects on head frequency, both causing reductions, it follows that both effects are to be referred to the changes they produce in the relative metabolic conditions that govern the regeneration of a head. As shown above, moving the level of section posteriorly tends to inhibit head formation, for, as the piece comes to involve the more posterior regions of the animal, those metabolic processes concerned in differentiation become increasingly less active. Reference to Figure 1 shows, however, that the fall in head frequency as the piece comes to involve the extreme posterior region of the animal is very abrupt, the frequency being about 4 per cent in one-eighth pieces. It was shown previously that in the intact adult animal the extreme posterior region is the site of most rapid growth (Buchanan, 1927). Unpublished data show also that this region is one of high susceptibility to poisons, a characteristic usually associated with relatively high metabolic rate. It seems probable, therefore, that as sections are cut to include more and more of this posterior active region as their posterior ends, the dominance of their anterior tissues tends to become less and less as the activity of their posterior tissues becomes greater and greater. This situation also makes for decreases in head frequency and seems to account for the abruptness in the fall of head frequency as the level of origin of the pieces approaches the posterior end of the animal.

The most probable explanation of the effect of the posterior cut of the piece on head frequency is that as the posterior cut is brought nearer to the anterior region of the piece, i.e., the pieces are cut shorter, the dominance of the anterior tissues of the piece in acting as leading elements in regulation becomes increasingly less, for as the posterior region of injury is brought nearer the anterior, greater proportions of the piece consists of tissues stimulated by injury. Thus the difference in metabolic activity that normally accompanies head development by anterior tissues tends to disappear.

It was noted that after a given interval following isolation the total length of regenerates is much the same regardless of the region of origin of the piece, but that the shorter the piece the greater the proportion of new tissue formed in regulation. The first of these facts demonstrates the equipotency of pieces that form a head. The second is in accord with evidence from other sources (e.g., Morgulis, 1911) that the quantity of tissue formed in regeneration is related to the severity of injury. Further interest attaches to the differences in distribution of newly formed tissues at anterior and posterior ends of the piece. Two types of metabolism are conspicuous in the regulative process, namely, differentiation and growth. Much evidence indicates that they may be antagonistic (e.g., Schmalhausen, 1930). Data already discussed show that differentiative metabolism decreases in rapidity and effectiveness as the level from which the piece is taken becomes more posterior. It was also shown that tissues from the same region differentiate a head if they form the anterior region of a piece, or differentiate a tail if they form the posterior region. Thus the type of differentiative process undergone depends on the position of the tissue with respect to the remainder of the piece. Similarly, the type of growth depends on the position of the tissue with reference to the remainder of the piece and in amount is apparently inversely related to the rapidity with which differentiation occurs. It is thus very certain that both differentiation and growth are controlled by factors residing in the piece as a whole and are not solely manifestations of properties inherent in the several tissues.

It will be at once evident that the explanation of regulation of pieces of *Phagocata gracilis* offered here does not differ in essentials from that proposed by Child (1916) for regeneration in pieces of *Planaria*, and subsequently adopted by the writer (1922). Child has preferred to express the conditions determining whether or not a head will form in a regenerating piece in terms of a ratio expressing the relation between the rates of metabolism of the cells of the anterior region and of the remainder of the piece. Thus, if the rate of metabolic activity of the tissues adjacent to the anterior cut surface is high as compared with that of the remainder of the piece, the head, the independent element of development, forms. If, however, because of the posterior position of origin of these tissues, or because of stimulation of the reminder of the piece, the metabolic activity is low in relation to that of other regions, the formation of a head does not occur. This interpretation best fits the facts at hand; it does not require the postulation of hypothetical agents and conditions but is based upon facts that are plainly evident in experimental data. It does not attempt to designate the particular types of cells involved in the formation of a blastema but accounts for the formation of that structure when tissues capable of forming a blastema are present. It leaves unanswered the problem of the nature of the physiological mechanism of control of a region of high metabolic rate over those of lower rate, but that such a controlling influence exists is hardly open to doubt. Nor does it attempt to answer the old, but still important, question as to why the tissues of some forms are capable of re-differentiating a new individual after they have been isolated from an adult, while this property is wanting in other, and in some cases related, forms.

SUMMARY

A cross-piece taken from any region of *Phagocata gracilis* either regenerates a normal head and undergoes complete regulation or does not regenerate at all.

In mass experiments the percentage of heads formed decreases sharply as pieces representing the same fraction are taken from more and more posterior regions.

Head frequency in mass experiments also decreases as the size of the pieces is reduced.

In pieces representing the same fraction of the animal the processes of differentiation and of growth are more rapid in anterior pieces than in posterior and are more rapid at the anterior than at the posterior end of the piece.

The length of new tissue regenerated anteriorly by a piece increases as the level of origin of the piece is moved posteriorly.

The length of new posterior tissue decreases as the level of origin of the piece becomes more posterior.

The total length of new tissue formed in regulation is approximately the same in pieces of the same size regardless of the region of origin of the piece but increases in proportion to the old tissue as the size of the piece is reduced.

When these facts are considered in their proper order, the conclusion is reached that whether or not a head forms in a piece removed from the adult body depends on the relation between the rates of growth and differentiative metabolism of the tissue adjacent to the anterior cut surface and that of other regions of the piece.

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THE LOCATION AND DIFFERENTIATION OF THE PRESUMPTIVE ECTODERM OF THE FOREBRAIN AND HYPOPHYSIS AS SHOWN BY CHORIO-ALLANTOIC GRAFTS[1]

(Two plates and three figures)

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THE original purpose of this investigation was the study of the location and capacity for differentiation of the presumptive hypophysial ectoderm. Data in regard to the capacity for differentiation of the hypophysis and, to some extent, of the ectoderm destined to form it have been reported previously (Stein, 1929). The results obtained at that time showed that the degree of organization was sufficient to cause the formation of a histologically normal hypophysis in chorio-allantoic grafts. This was true in the majority of cases from sixteen somite and older donors and in a few cases from younger donors. The donors used for the previous experiments ranged in age from the headfold stage to 5 days. Comparatively few grafts were made of implants from young donors, headfold and early somite stages. It was therefore thought advisable to extend the work to include these stages, and also the head-process stage, in order to have a more complete story of the location and the capacity for differentiation of the ectoderm which later forms the anterior lobe of the pituitary gland. As in the previous paper, the term "hypophysis" is used to designate the ectodermal structure otherwise known as "Rathke's pocket."

Hunt (1931*a*) suggested that the medullary plate may depend on the notochord for its differentiation. Certain of the grafts made in the course of this work (of negative value as far as the hypophysis was concerned, since the latter failed to differentiate) seemed, however, to shed some light on the questions of the origin and determina-

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[1] It gives me pleasure to express here my appreciation for the helpful suggestions and criticism of Dr. B. H. Willier, who recommended the problem and under whose direction the work was carried on.

tion of the forebrain tissue. Consequently additional grafts were made for the study of these problems.

This paper therefore presents the data obtained from two more or less independent series of experiments. In regard to the forebrain it deals with the location of the tissue giving rise to that structure, with the capacity of that tissue for differentiation when isolated from head-process and from node material, and with the question of the probable time of its determination. The hypophysis and presumptive hypophysial ectoderm are considered in the light of their capacity for differentiation and of the location of the latter.

MATERIAL AND METHODS

Eggs of white Leghorn chickens were used for all the experiments. The method of grafting was that described by Willier (1924) with the exception of slight modifications. A very fine pipette with approximately an inch of the tip bent perpendicularly to the stem was used for transferring the implants to the chorio-allantoic membrane of the 9-day-old host. Solution was first taken into the pipette until the tip remained full even when no pressure was exerted on the bulb. By slight pressure a drop was then ejected and a drop containing the implant taken in. The tissue thus remained at the tip of the pipette during transfer and could be ejected with a minimum amount of fluid by simply touching the tip of the pipette to the chorio-allantoic membrane. Grafts were removed on the sixteenth, seventeenth, or eighteenth days of incubation. All were fixed in Bouin's fluid, sectioned serially at 8 μ, and stained in Heidenhain's iron haematoxylin or, in a few cases, in Ehrlich's haematoxylin and eosin.

For the study of the forebrain the donor embryos used were of primitive-streak, head-process, and early headfold stages. Since number of hours of incubation is not an accurate criterion for judging the actual stage of development of such young embryos, the groupings have been made according to the length of the primitive-streak or head-process. At the time of operation the blastoderm was removed from the yolk and placed in a Petri or Syracuse dish of 0.9 per cent salt solution warmed to 39° C. Measurements were then taken with a Zeiss ocular micrometer which was graded in tenths of a millimeter. Under the Spencer binocular dissecting scope used, with

32 mm. objective and 6× ocular, one unit represents 0.033 mm. Measurements have been expressed in units in the tables, in millimeters in the text of the paper. Knife-bladed needles were used for cutting the blastoderm and after cutting measurements were taken in most cases of the distance of the cut from Hensen's node, the primitive pit, or the head-process, as the case might be. These were approximate measurements, since exact boundaries of these structures are not always clearly distinguishable. There was found to be a great amount of variation among donors in this respect, and for the most part embryos in which structures were not comparatively distinct were discarded. The types of implants made in the three series of experiments on the forebrain are illustrated in Figures 1, 2, and 3 and are described with the results obtained in each case in the section following.

The embryos used for studying the differentiating capacity of the ectoderm destined to form the hypophysis were of head-process, headfold, and early somite stages. The experimental procedure was the same as that described for the experiments on the forebrain. From donors of presomite stages all the material anterior to Hensen's node was implanted, from early somite stages either all anterior to the first somite, or in some cases all the tissue ventral to the gut cavity plus a varying amount of the forebrain. A series of grafts was also made from head-process stages in which the implants extended from the anterior opaque area to include a varying amount of the head-process. Because of the failure of hypophysis to differentiate in these grafts, with one exception, they are not described in detail in this paper.

DATA OBTAINED FROM GRAFTS

THE CAPACITY OF THE FOREBRAIN FOR DIFFERENTIATION

Grafts from implants of head-process stage. Series I.—The first problem to be investigated in regard to the forebrain was whether it would differentiate after isolation of prospective forebrain material during the head-process stage from all posterior and especially all notochordal material. Embryos of head-process and very early headfold stages were used for the first series of experiments. Figure 1 shows the types of implants. Type *a* consisted of most of the material anterior to the anterior tip of the head-process up to the opaque area. Type *b* included the material behind *a* either back to the node level or including the node level. From eighty-five live hosts which received Type *a* implants and were alive at the time of opening, only twenty-nine grafts were obtained. Table I gives the list of structures differentiated in twenty-six of the Type *a* and twelve of the Type *b* grafts, together with measurements made before implantation. The twelve Type *b* grafts are from the same donors as twelve of the Type *a* grafts.

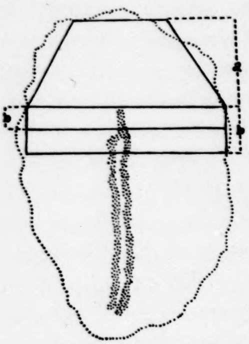


FIG. 1.—Outline showing implants for Series I.

The three remaining grafts, of the twenty-nine mentioned above, were not listed in the table because the only tissue they contained consisted of a few vacuolated epithelial tubes and scattered cells. It was thought at first that these tubes were endodermal in origin, but later grafts of ectoderm or ecto-mesoderm alone were found to contain structures identical in appearance (see Table II*). Therefore it has been concluded that the vacuolated cells came either from material destined to be skin epithelium or brain. An ectodermal origin seems more likely than a mesodermal one for various reasons. Pigment granules are present in similar tubes in two grafts of Series I (100–9, 87–9). In the latter they form part of the pigmented coat of the retina which, together with other forebrain tissue, is differentiated in this graft.

It will be seen from Table I that forebrain almost invariably differentiated from grafts of material anterior to the head-process or notochord, that is, from Type *a* implants, in head-process and early headfold stages. This was true even when material anterior to the apparent anterior end of the head-process was included in the Type *b* implant, as is known by measurements for some of the grafts. Even where measurements were not recorded of the distance of the cut from the anterior end of the head-process, care was taken not to cut too close to the visible end, since the head-process ends in fusion with the prechordal plate material. The latter was very seldom distinguishable, but from the almost invariable presence of mesodermal structures such as cartilage and bone it seems necessary to conclude that part of it was included in the Type *a* implants.

The complete absence of notochord in the grafts from Type *a* implants suggests that none of the head-process material was included in such implants; but it is, of course, possible that an amount of material too small to differentiate or persist in a graft might be enough to induce the formation of nervous tissue if such induction is necessary. That notochord does not always differentiate even when head-process or node material was known to have been included in the implant can be seen from the data on Type *b* implants in Tables I, II, and III. The following arguments are thought more conclusive in establishing the non-inclusion of head-process tissue in the Type *a* implants. Wetzel (1929) has shown that the material anterior to Hensen's node in primitive-streak stages gives rise to the forebrain and that the anterior end of the head-process in later stages represents the original position of the node. The presence of eye in grafts from both *a* and *b* implants from the same donor, which

TABLE I†

STRUCTURES DIFFERENTIATED IN GRAFTS FROM HEAD-PROCESS DONORS. SERIES 1

Number of Grafts	Type	Length of Head-Process in Units	Distance of Cut from Node in Units	Number of Grafts in Which Structures Listed Appear														
				Brain Tissue	Prosencephalon	Mesencephalon	Eye	Epiphysis	Choroid Plexus	Ganglia	Epidermis	Notochord	Cartilage	Bone	Heart	Skeletal Muscle	Gut	Endodermal Glands
6	*a*	1–4	4–7	6	5	...	4	1	3	...	4	...	4	1	1	2	5	2(1T, 1L)
3	*b*			3	1	...	1	...	...	1	2	1	2	...	1	...	3	...
7	*a*	5–14	6–14	6	5	...	5	...	2	...	4	...	5	2	2	1	7	5(5T, 2L)
2	*b*			2	1	1	1, 1?	1	2	2	2	1	2	...	1	1	2	1T
9	*a*	15–22	18–25	8	6	...	6	...	3	...	5	...	4	1	...	2	7	5(4T)
5	*b*			5	5	2	5	1	2	2	5	2	5	2	3	4	5	4(3L)
4	*a*	Early head-fold	.....	4	1	...	1	...	1	...	2	...	3	1	...	...	3	2T
2	*b*			2	2	2	2	...	2	2	2	1	2	2	1	2	2	2(1T, 1L)

† KEY: *a* = grafts of implants anterior to head-process, *b* = of head-process level, *T* = thyroid, L = liver.

occurred five times in this series, would therefore indicate that head-process material was not included in the *a* implants; yet the brain tissue developed typically in such grafts. The case of a special graft (101–9) may also be mentioned here. For this graft measurement was made not only of the distance of the cut anterior to the head-process but also of the amount of medullary plate material included in the implant. This was found to be approximately 0.16 mm., or a little less. In the living blastoderm the area of the primordium of the medullary plate anterior to the node or early head-process measures between 0.32 and 0.47 mm. in anterior-posterior direction. This approximates Wetzel's measurements of the same area as far as could be told from scale drawings and reconstructions. It would seem quite conclusive, therefore, that no head-process material was included in the implant of the above-mentioned graft; yet again brain and eye differentiation were typical.

The histology of the grafts from head-process and headfold stages is summarized with that of primitive streak implants on page 213 and is figured on Plate I (Fig. 4).

Grafts from implants of primitive-streak stage. Anterior and node levels. Series II.—From the data of grafts of the head-process stage it seemed possible that the presence of prechordal mesoderm might be sufficient to induce brain formation if induction is necessary. For this reason a series of grafts of definitive primitive-streak stages was made. As shown by Wetzel (1929) and Adelmann (1922), prechordal mesoderm is present anterior to Hensen's node in these stages. The *a* implants of Series II were similar to those made by Hunt (1931*a*), including the area between the node and the anterior limit of the pellucid area (see Fig. 2). Three grafts (94–15, 95–3, and 105–13) were from implants of ectoderm or mesectoderm only. Two of these are indicated (by an asterisk) in Table II; the third contained only epithelium and fibrous tissue. The only difference in technique from Series I was that measurements were taken of the length of the primitive-streak and of the distance of the cut from node and pit. Type *b* implant for this series included the node level only. See Figure 2 for the types of grafts. From forty-seven live hosts twenty grafts were obtained of which twelve, and their reciprocals where recovered from *b* implants of the same donors, are summarized in Table II as to the structures which differentiated. The other eight grafts were like the six of Series I mentioned above, containing only vacuolated epithelial tubes. Of these eight, two were from implants of ectoderm or ectomesoderm only, the endoderm having been removed very easily as a continuous layer before grafting. As noted by Hunt (1931*a*), the endoderm separates very readily from the ectoderm; and in some cases complete separation occurs without the efforts of the operator. This separation can always be noted along the posterior cut edge of the *a* implant; and, as in Hunt's operations, a bit of the opaque area was sometimes included in such implants in order to insure the adherence of the germ layers to each other.

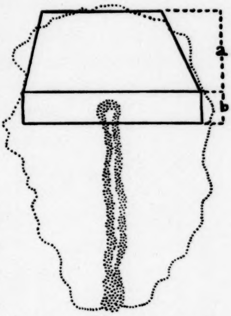


FIG. 2.—Outline showing implants for Series II.

The amount of material differentiating in grafts of Type *a* implants of this series was slight. As can be seen from the table, epithelium was the most constantly recurring structure. Practically all of the grafts included in the table showed tubules of vacuolated epithelial cells like those of the eight grafts mentioned above. Their probable origin has been suggested under Series I. Brain tissue could be positively identified in only three cases (see Fig. 5), in one of which it was represented solely by a small mass of the pigmented coat of the retina. Its differentiation corresponded to that of brain in grafts of head-process stages. Masses of unidentifiable tissue which it was thought might be nervous tissue (indicated in Table II by a question mark) were present in several cases (see Fig. 6). The similarity of this tissue to that found at the edges of brain vesicles in grafts from older donors suggested this interpretation. It may be noted here that the three cases of actual differentiation of brain tissue, and two of the three of tissue resembling nervous tissue, appeared in grafts from donors with primitive-streak lengths in the range of 2.16–2.76 mm., 60–80 units. The length of the primitive-streak at the time of the origin of the head-process differs in different individual blastoderms but approaches the range of 2.00–2.76 mm. This statement is made on the basis of measurements of forty embryos in which the head-processes had just begun, being 0.16 mm., or less, in length. Three of the forty had primitive-streaks only 1.83 mm. long, eighteen were between 2.00 and 2.33 mm., sixteen between 2.33 and 2.50 mm., one 2.76 mm., and two 3.00 mm.

Grafts from implants of primitive-streak stages. Antero-lateral and node-streak isolations. Series III.—A third series of experiments, made like the second on primitive-streak stages, gave comparable results. In this series the Type *a* graft contained a larger area of prospective medullary plate material than the Type *a* graft of Series II. Node and streak material was excluded, but most of the material up to the opaque area and all lateral to the streak for the anterior half of its length was included. The *b* implant included the node, a bit of the tissue just anterior to it, and approximately the anterior half of the primitive-streak. Figure 3 illustrates the type of cut made, while Table III lists the structures which differentiated from both Type *a* and Type *b* implants and gives the measurements made at the time of operation.

TABLE II†

STRUCTURES DIFFERENTIATED IN GRAFTS FROM PRIMITIVE-STREAK DONORS. SERIES II

Number of Grafts	Type	Length of Primitive-Streak in Units	Distance of Cut		Number of Grafts in Which Structures Listed Appear															
			From Node	From Pit	Brain Tissue	Prosencephalon	Mesencephalon	Eye	Epiphysis	Choroid Plexus	Ganglia	Epithelium	Fibrous Tissue	Notochord	Cartilage	Bone	Heart	Skeletal Muscle	Gut	Endodermal Glands
6	*a*	40–55	1–6	3½–8½	*1?	..	..	..	..	..	..	6	4	..	..	..	..	..	2?	....
1	*b*				1	..	..	..	..	..	1	1	..	1	..	1	1	1	1	1(1T, 1L)
6	*a*	60–80	2½–6	4½–8	3+2?	*1	..	*1	..	..	..	3	1	..	..	..	..	..	4	....
3	*b*				3	2	1	2	1	2	2	3	..	2	2	..	1	2	2	2

† KEY: *a* = grafts of implants anterior to node, *b* = of node level, T = thyroid, L = liver.

* Implant for graft contained no endoderm.

It is again noticeable that, with one exception, the Type *a* grafts in which nervous tissue appeared were from donors with fairly long primitive-streaks, between 2.16 and 2.83 mm. Apparently a certain stage was usually reached in all grafts of implants from primitive-streak donors before differentiation of brain tissue would occur in the absence of node and streak material. The one exception to this is the case of the graft 99–11, which contained a small amount of brain tissue, and for which the implant came from a blastoderm with a primitive-streak approximately 1.6 mm. in length. It had been noted at the time of operation that the cut had been made very close to the edge of the streak some distance below the region of the node. In the case of one Type *a* graft (78–3) a small bit of tissue resembling notochord appeared. Since the donor for this graft had a primitive-streak of 2.48–2.64 mm., it may be that the head-process had begun to form and that the tip of it was included in the implant.

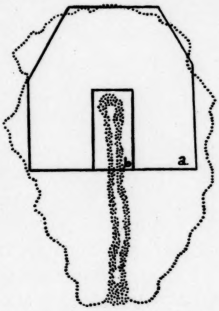


FIG. 3.—Outline showing implants for Series III.

It was not possible to determine whether the brain present in these grafts was fore-, mid-, or hindbrain except when eye or epiphysis or the stratification typical of midbrain appeared. The percentage of takes from Type *a* implants of this series was better than of Type *a* implants in Series I and II, where only the material anterior to the head-process or to Hensen's node was included (see Table IV). This was thought due to the larger amount of material included in Type *a* implants of the present series, a condition which perhaps allows vascular connections to be more readily established at the time of implantation.

THE HISTOLOGY OF GRAFTS OF SERIES I, II, AND III

The histology of the tissues in these grafts is essentially the same as that described by Willier and Rawles (1931) from implants of total blastoderms of the same ages. The chief differences between grafts from implants of anterior or anterior and lateral material (Type *a*) and those including node level or node and streak (Type *b*) are that a smaller number of tissues and a lesser amount usually differentiates in the former and that the arrangement thereof is

PLATE I

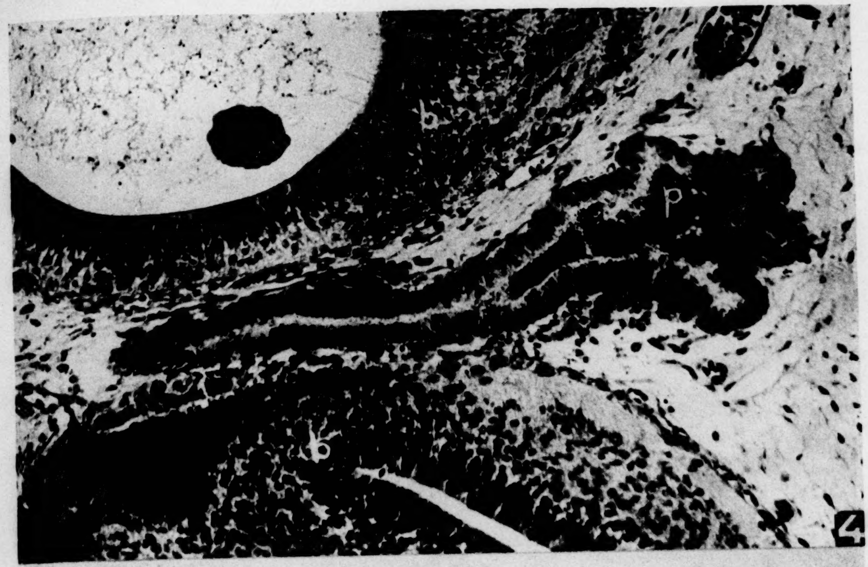
b=brain, *e*=epidermis, *h*=hypophysis, *n*=nervous tissue, *p*=pigmented layer of retina, *v*=vacuolated cells.

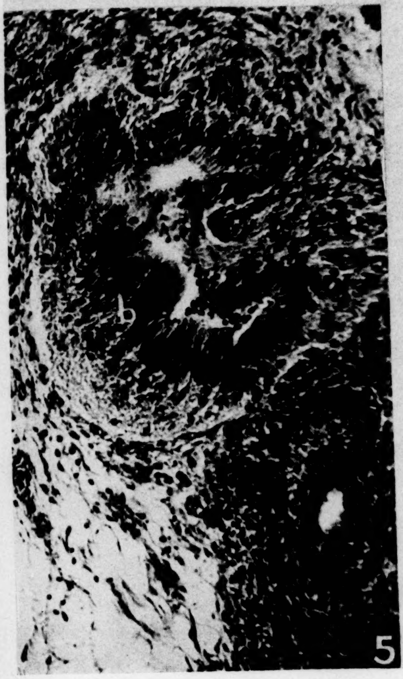
FIG. 4.—Photomicrograph of section of graft 101–9. Donor with head-process of approximately 0.03–0.06 mm. Implant from level anterior to head-process (Series I, Type *a*). Age of grafted tissue, 8 days. ×210.

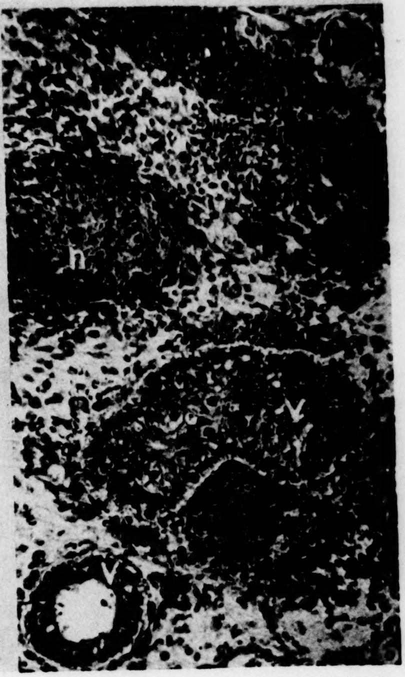
FIG. 5.—Photomicrograph of section of graft 92–7. Donor of primitive-streak stage. Implant from level anterior to primitive-streak (Series II, Type *a*). Age of grafted tissue, 8½ days. ×210.

FIG. 6.—Photomicrograph of section of graft 95–3. Donor of primitive-streak stage. Implant of ecto-mesoderm anterior to primitive-streak (Series II, Type *a**). Age of grafted tissue, 8½ days. ×210.

PLATE I







usually less normal, rather than that the degree of differentiation attained is lower. This is true also for a comparison of the grafts of primitive-streak donors as compared with those of head-process donors.

TABLE III†

STRUCTURES DIFFERENTIATED IN GRAFTS FROM PRIMITIVE-STREAK DONORS. SERIES III

Number of Grafts	Type	Length of Primitive-Streak in Units	Type *b* Implant		Distance of Cut Anterior to Node in Units	Number of Grafts in Which Structures Listed Appear														
			Length	Width		Brain Tissue	Prosencephalon	Mesencephalon	Eye	Epiphysis	Choroid Plexus	Ganglia	Epithelium	Notochord	Cartilage	Bone	Heart	Skeletal Muscle	Gut	Endodermal Glands
7	*a*	40–55			2–4	*1	..	..	..	..	..	..	7	..	..	..	3	1	7	....
4	*b*		20–35	10–16		2	..	1	..	..	..	..	4	1	..	..	2	1	3	1L
13	*a*	60–85			2–5	7+2?	3	..	3	2	3	..	12	1	3	..	7, 1?	1	12	4(2T, 3L)
4	*b*		30–40	13–16		2	..	1	..	..	..	..	2	..	3	..	2	..	4	1T

† KEY: *a* = grafts of implants anterior and lateral to node and streak, *b* = of anterior half of streak including node, T = thyroid, L = liver.

* Implant cut close to streak.

TABLE IV

COMPARATIVE DATA FOR TYPE *a* IMPLANTS OF SERIES I–III

Stage of Donor	Type of Grafts	Number of Hosts Alive	Total Number of Grafts Obtained	Number Containing Nervous Tissue or Tissue Similar Thereto	Percentage of Grafts Obtained	Percentage Containing Nervous Tissue or Tissue Similar Thereto
Head-process....	Anterior to head-process, Series I, Type *a*	85	29	24	34.1	82.8
Primitive-streak.	Anterior to node, Series II, Type *a*	47	20	6	44.7	30.0
Primitive-streak.	Anterior and lateral to node, Series III, Type *a*	28	20	10	71.4	50.0

PLATE II

b = brain, *e* = epidermis, *h* = hypophysis, *n* = nervous tissue, *p* = pigmented layer of retina, *v* = vacuolated cells.

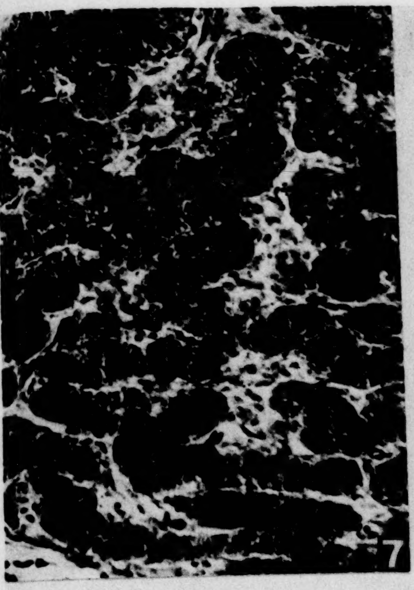
FIG. 7.—Photomicrograph of hypophysial cords of normal chick embryo of 12 days' incubation. ×275.

FIG. 8.—Photomicrograph of section of graft 5–20 showing hypophysial cords. Age of donor, 3 days; of grafted tissue, 13 days. ×275.

FIG. 9.—Photomicrograph of section of graft 19–7. Age of donor, 12 somites; of grafted tissue, 9½ days. ×275.

FIG. 10.—Photomicrograph of section of graft 82–10. Age of donor, head-process stage; of grafted tissue, 10 days. ×275.

PLATE II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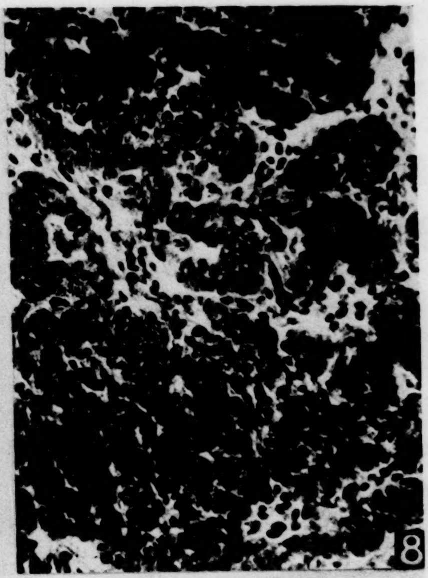
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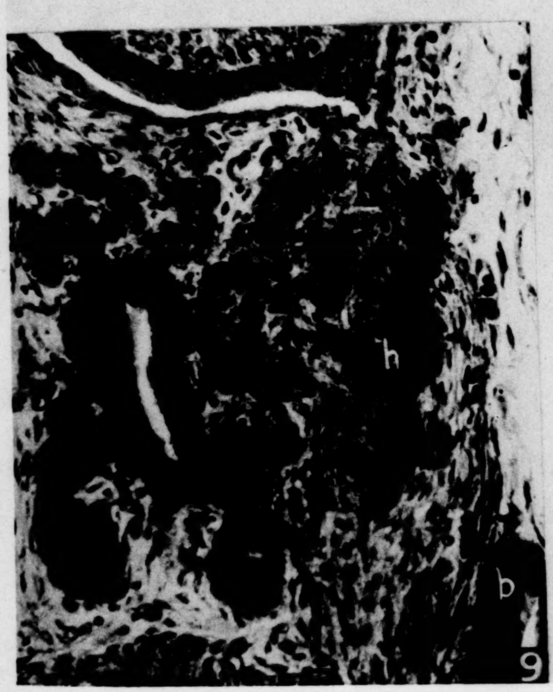
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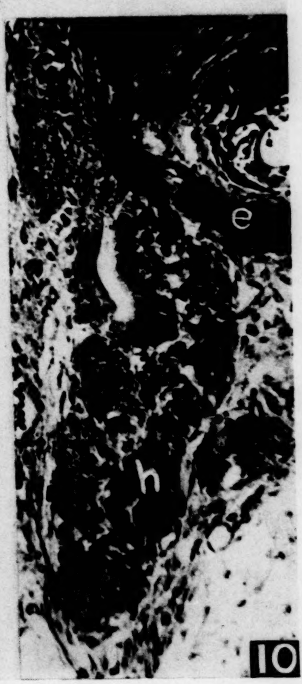
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Figures 4, 5, and 6 give some idea of the amount of differentiation attained in Type *a* implants. The brain occurs typically in the form of vesicles with a definite lumen bounded by columnar-shaped cells. The internal limiting membrane is in almost all cases distinct; and between it and the first nuclei there is a narrow non-nucleated margin, the internal ependymal layer, the width of which is approximately uniform around the lumen. Mitoses are usually fairly numerous in the nuclei lying closest to the lumen. The cells lining the lumen give an appearance of pseudostratification; their cytoplasm is comparatively dense, being bluish gray in color with the stain used. External to the area just described is a looser-looking area which varies greatly in width in different grafts and in different regions within the same graft. Variation in width of this layer is also found in the normal chick. Here the nuclei are fewer in number and are scattered, seemingly connected with fiber-like strands of cytoplasm. The general appearance of this area is spongy or veil-like. In some cases more definite fibers appear to be present. It is in this area that signs of disintegration are most often found in the grafted tissue. It may be heavily infiltrated with blood cells or may look as if eaten away, large holes being present with only a few strands of tissue and a few isolated nuclei separating them. Outside this area there may or may not be a definite external limiting membrane. Sometimes the tissue appears to be unconfined and to lose itself among the strands of chorio-allantoic tissue or that of other structures in the grafts. Compared with the forebrain of a normal chick of 9 days' incubation, the latter is found to have the same layers; but the cells seem smaller and much more closely packed. In the veil-like layer in the normal brain the fibers are perhaps more distinct—the structure not quite so spongy; this may also be simply a matter of fewer cells in the brain tissue of the graft. Comparison with the normal in the case of the brain is not especially helpful in determining the degree of delay in differentiation caused by the implantation, since the histological structure of the forebrain of chicks of 7–9 days is essentially similar.

As far as eye is concerned, the pigmented layer of the retina is sometimes the only part appearing in grafts from implants excluding all node and streak (Type *a* in all three series). It is, however, present in all cases where eye is recorded. The pigmented layer in some cases is a single row of cells which vary in shape from cuboidal to low columnar. Sometimes it is very much twisted, owing probably to abnormal growth factors in the graft; and in these cases the mass of it may appear to bud off small tubes which may, in some grafts, be cut in cross-section. In all cases the cytoplasm is literally packed with dark-brown or black pigment granules, so that cell outlines and often nuclear outlines cannot be distinguished. The difference from the normal of the same age is that in the graft retina the cells seem larger and less numerous, and their arrangement in foldings and masses of tissue is abnormal.

The sensory layer of the eye, when present, corresponded to that in the normal embryo of 8 days of incubation. The grafted tissue was between 9½ and 10 days of age. Thus there was a delay of approximately 2 days in the eye of the graft, and this delay was measured by the absence of the outer molecular layer. The inner and outer nuclear layers were thus fused except that in places a small light line, evidence of the beginning separation, was present. This condition is also present in areas of the sensory layer of the retina of the 8-day chick.

The ectodermal epithelium found in the grafts was sometimes stratified and sometimes resembled oral and nasal epithelium. No attempt was made to identify the various types in the individual grafts, since it did not seem necessary for the purposes of this investigation. The vacuolated type of epithelial tubes has already been mentioned. Some grafts of all three series showed stratified epidermis and feather germs, the latter being in general better developed in grafts of implants of head-process stages than in the corresponding implants from primitive-streak stages.

The endodermal glands were identified by their connection with gut tissue. The latter consisted of tubes of cells with characteristically darkly staining nuclei sometimes surrounded by a band of muscular or fibrous tissue. Individual identification of pharynx and various gut levels was not made, although they were differentiated in some of the grafts. No evidence of trachea, with its characteristic arrangement of cartilage, was noted. Thyroid and liver were distinguished by a series of criteria; the level of gut with which they were connected, the presence of colloid which is sometimes found in the thyroid, and the darkly staining nuclei and vacuolated cells characteristic of the liver, as well as by the differing arrangement of the cords of cells which are more solid and more infiltrated with blood in the case of liver than of thyroid.

Notochord did not occur with great frequency, but was found in contact with nervous tissue when it did occur. It had the typically vacuolated structure characteristic of its appearance in the normal embryo. Cartilage and bone were found in most of the grafts with the exception of those in Series II from Type *a* implants. Heart, where present, was usually a mass of cardiac tissue which possessed a lumen in a few cases only.

THE DIFFERENTIATION OF THE HYPOPHYSIS

Previous work (Stein, 1929) has shown that the hypophysis will differentiate in grafts from embryos ranging in age from 5 days to the headfold stage. Table V is a summary of grafts made for the previous study and of additional grafts of implants from headfold and head-process stages recently made.

The amount of tissue included in the implants varied according to the age of the donor. For the older embryos this was reported in the 1929 paper. For the younger, it has been given earlier in this paper.

The percentage differentiation of the hypophysis decreases steadily with the age of the donor, as can be seen by consulting Table V. A most noticeable drop, from 80 per cent to 55 per cent, comes between the fifteen- to twenty-two-somite and the eight- to twelve-somite stages. This coincides very nicely with the first appearance of the hypophysis as a thickening of the ectoderm, which, according to Adelmann (1922), occurs in the fifteen-somite embryo.

The structure of the hypophysial cords in grafts from all stages of donors is essentially the same. They are two cell layers in width in their thinner portions, and three to four where they branch or curve on themselves. Measurements with an ocular micrometer show that in the grafts of all stages and in normal 11- and 12-day embryos these cords are actually the same width. This is also evident for the grafts from the Figures 8, 9, and 10. No cytological differences are evident, but these would not be expected in tissue of this age. The great difference between hypophysis in grafts from younger and those from older donors is in the amount of tissue present. As would be expected, this is less in grafts from younger donors. What makes the difference even more striking is that where there is a small amount of hypophysial tissue the appearance of the gland as a complexly branched structure is altered to that of a few cords with budlike projections, or to an aggregation of small lumplike masses of tissue, depending on the section. This results in there being fewer spaces for mesenchyme and blood capillaries and so further alters the picture characteristic of the gland in the normal embryo.

TABLE V

DATA ON THE CAPACITY OF THE HYPOPHYSIS FOR DIFFERENTIATION

				NUMBER OF GRAFTS IN WHICH STRUCTURES LISTED APPEAR																
AGE OF DONOR†	NUMBER OF GIFTS	HYPOPHYSIS	INFUNDIBULUM	Brain	Epiphysis	Eye	Lens	Choroid Plexus	Ganglia	Epidermis	Epithelial Ingrowths	Feather Germs	Skeletal Muscle	Heart	Cartilage	Bone	Notochord	Gut	Thyroid	Liver	Percentage of Appearance of Hypophysis
3–5 days...	16	15	15	16	2	4	..	7	10	16	10	15	14	..	16	16	4	....	....	..	94
23s–54 hr...	9	7	6, 3?	9	..	7	1	1	..	7, 1?	6	3	7	..	9	6	....	1, 2?	....	..	78
15s–22s.....	10	8	7, 1?	10	..	7	3	...	2	8	7	5	7	..	9	8	....	1	1	..	80
8s–12s......	9	4, 1?	2, 4?	9	1	9	6	2	..	7	4	4	4	1	8	5	....	1	1	..	55
1s–7s......	15	3	1, 3?	15	3	12	3	6	1	15	6	10	5	3	12	5	1	9	4	1	20
Headfold and head-Process...	20	2, 1?	2	20	..	11	1	12	5	20	2	8	9	10	17	9	5, 2?	19	10	9	15

† Somites are indicated by "s."

Hypophyses from donors up to fifteen somites are in all cases connected with oral epithelium, and some of them still show a large central cavity from which the cords bud. This is thought to be the remains of the lumen of Rathke's pocket. The connection with oral epithelium was not present in a 9-day-old normal embryo, but its presence in the 10-day and older grafted tissue may be the result of mechanical conditions preventing normal growth relations in the grafts rather than an evidence of retardation. This may also be true for the remains of the lumen of Rathke's pocket, or the presence of the latter may be evidence of a real retardation. It is present in the 7-day-old normal embryo but has disappeared by 9 days, while the grafts in which it was present were 9–10 days in age of grafted tissue.

The grafts in which the hypophysis has not differentiated seem to have no other common factor with which the absence of the hypophysis could be correlated. Four grafts of the somite stages show no differentiation of epidermal structures; but the remaining thirty-one grafts which lack hypophysis have well-differentiated epidermis, and eight of these, scattered through a range from head-process to 4-day donors, have cordlike outgrowths (not hypophysial) from epithelium similar to oral epithelium and connecting with stratified epidermis. Fifteen have easily recognizable feather germs; five have a lens associated with eye tissue. Consequently, in most of the cases skin ectoderm was differentiating, as far as other structures than hypophysis were concerned. All the grafts have brain tissue; and, since most of them have pigmented cells of the retina, this was interpreted as diencephalon. The actual identification of an infundibular process is felt to be unreliable in the absence of hypophysis. There were in some cases brain outgrowths which resembled those occurring in the region of the hypophysis when the latter was present in grafts, but they could not be specifically recognized as infundibular. On the other hand, hypophysis, while always found in close relation to brain tissue, was not always associated with a typically normal infundibular process.

The amount of tissue included in the implant did not seem to have any connection with the presence or absence of hypophysis in the grafts. For all of the head-process and headfold stages summarized in Table IV the implant included all the tissue anterior to Hensen's node. However, another graft, 47–8, for which the implant extended posteriorly to include only the anterior half of a head-process about 0.6 mm. long, contained well-differentiated hypophysis. The implants for the three grafts containing hypophysis from one to seven somite donors included only the tissue ventral to the gut cavity and the ventral part of the forebrain. The other grafts from donors of the same stage, though from implants containing the same amount of tissue in four cases and including also the dorsal forebrain in six, plus the midbrain in two cases, had no hypophysis present in them. The grafts showing non-differentiation from older donors were in all cases from implants identical in amount of tissue with those which furnished hypophysis in other grafts.

DISCUSSION

THE LOCATION OF FOREBRAIN MATERIAL

Wetzel (1929), in a series of vitally stained embryos, found that the material giving rise to the forebrain lies anterior to Hensen's node in the primitive-streak stage, and the anterior-posterior length of the area would seem from his measurements to be not more than 0.4–0.5 mm. In his vitally stained embryos, if the stain was placed anterior to the node in the definitive primitive-streak stage, the color appeared in the forebrain; if the node level alone was colored, the forebrain remained unstained but the notochord and more or less of the neural tube posterior to the forebrain was stained. The former type of experiment used by Wetzel, that of placing the stain anterior to the node, was repeated by the present writer on a small number of embryos. The technique of Kopsch, confining the agar in very fine glass tubes which could be held against the blastoderm and a fairly distinctly localized mark obtained, was used. The results agree with those of Wetzel, since forebrain was found to be stained in the four cases in which development had been normal and the stain was deep enough to determine its location at the time of examination.

The morphological studies of Kingsbury and Adelmann (1924, pp. 247–48) have brought them to the conclusion that among vertebrates the prechordal medullary tube "furnishes the 'first primary brain vesicle' from which the optic vesicles arise by a continuation of the bilateral expansion." Kingsbury (1920, 1922, 1931) considers that the epichordal brain, as opposed to prechordal, extends from the medial floor of the midbrain backward. The chick embryo was one of the types of vertebrate embryos used by him in this study.

In this paper it has been shown that forebrain will differentiate in chorio-allantoic grafts in almost every case from implants of the material anterior to the head-process, and in several cases from implants of the material anterior to the node in the stage just before or about the time the head-process has begun to form. These results are thus in line with the idea that the forebrain material, including eye material, is located anterior to the head-process in the normal chick embryo of that stage, and anterior to Hensen's node in the definitive primitive-streak stage. The experiments are not suitable for defining the posterior limit of the forebrain material, since in the attempt to insure exclusion of the tip of head-process or node from the implants the cut for isolation was usually made somewhat anterior to the apparent anterior limit of these structures.

This presence of some anterior material in the implant may explain the presence of forebrain as denoted by eye or epiphysis in many of the grafts from the posterior level which included also either all of the head-process anterior to the node, or the node level, or both, depending on the stage used (level *b* in all grafts). This explanation would also seem adequate for the presence of forebrain structures in Hunt's grafts of the node level of definitive primitive-streak blastoderms. It does not account for the presence of hypophysis reported by Hunt in the same grafts, if, as seems likely, the hypophysis material is located anterior to that giving rise to forebrain. The absence of hypophysis from eleven grafts of implants of the same level made by the writer and its rare occurrence in grafts of all the material anterior to the node from later stages up to nine somites suggests that the structure identified as hypophysis by Hunt may be something else. Thyroid sometimes has a similar cordlike appearance in grafts, and Hunt makes no record of the appearance of the latter structure. In a previous paper (Stein, 1929) the author reported hypophysis as present in a graft of a presomite stage. This has since been found to be thyroid. Hunt has also stated (personal communication) that the hypophysis recorded in his grafts was not always in close relation with the brain. From entire blastoderms Willier and Rawles (1931) obtained only three cases of hypophysis in twenty-eight grafts of primitive-streak and head-process stages, while Hunt reports seven cases out of twenty-five in grafts of the node level alone of the definitive primitive-streak stage. Even if hypophysis is present in some of these cases, the possibility of its being the result of induction by the forebrain material is not disproved.

Therefore, in any case, Hunt's material does not exclude the possibility of forebrain material being definitely located in front of Hensen's node in the primitive-streak stage. It may, of course, be possible that the removal of the anterior level stimulates the level behind it, the node level, to form anterior structures for which the material was lacking in the implant. This would mean that the node level is more or less totipotent. However, Hunt's later experiments (1931*b*) in transecting the node and implanting parts of it to the chorio-allantoic membrane suggest that the node, rather than being totipotent, is in itself a mosiac, as indicated by the fact that the anterior half of the node level gave anterior structures only, the posterior half posterior ones. The first explanation for the presence of forebrain structures in both my grafts and those of Hunt from implants of the node level—viz., that a part of the material anterior to the node and normally giving forebrain was included in such implants—would therefore seem the more likely one.

It may be mentioned here that the presence of a structure in a graft does not mean that the structure is necessarily present in its entirety, that is, for example, that a complete forebrain or eye is present in the foregoing grafts. Histologically, of course, it is possible to say that forebrain tissue, pigmented and sensory coat of the eye, etc., are present; but there is no reliable criterion at the age attained by the grafts for judging whether or not part or all of the telencephalon is present. Shape is too abnormal in most cases; and the amount of material is not as great as that attained in the normal embryo. The latter is true even for grafts from implants which included the entire primordium of a structure from a later donor, as, for example, in the experiments of Hoadley (1924) on the eye, nasal region, otic region, and mesencephalon. Hoadley (1929) has pointed out that differentiation of structures in chorio-allantoic grafts takes place regardless of the retardation of cell multiplication.

Taking into account all the facts discussed above, there seems to be definite positive evidence for the localization of forebrain material anterior to Hensen's node in the definitive primitive-streak stage of the chick embryo. Also, as far as I have been able to discover, there is no positive evidence against this conception.

THE DETERMINATION OF FOREBRAIN MATERIAL

It has been suggested by Hunt (1931*a*, p. 423), in his study of the rôle of Hensen's node in the development of the chick embryo, that "the head-process induces the medullary plate to form in the chick." From a study of grafts from implants of the entire pellucid area of blastoderms ranging from the definitive primitive-streak stage to the eleven-somite stage, Willier and Rawles (1931, p. 460) state that it "is somewhat well brought out" in such grafts "that the notochord may be of importance in bringing about the development of the medullary plate." They note that, "as a rule when any part of the central nervous system, except the forebrain, differentiates, it is in more or less intimate association with notochord." This is, of course, the normal situation, since the notochord in the chick embryo extends approximately up to the level of the infundibulum, separated from the latter, according to Kingsbury (1922), by the extent of the prechordal plate material. As has previously been discussed, the localization of forebrain material in the definitive primitive-streak stage seems to be anterior to Hensen's node; and this corresponds with its position anterior to the notochord in later stages, since, as shown by Wetzel (1929), the position of Hensen's node in the primitive-streak stage is that of the anterior end of the notochord in later stages. The present experiments of implanting to the chorio-allantoic membrane material anterior to the head-process stages have shown that the forebrain material will differentiate after isolation from all notochord or head-process material. This is true even for implants of the anterior material from embryos in the earliest recognizable stage of the head-process. Therefore, it would seem that development of the forebrain is not dependent on the presence of notochord or head-process after the latter has become visible.

The differentiation of forebrain from the level anterior to the node in the definitive primitive-streak stage occurred normally in only three grafts (Series II); and in only one of these, in which the pigmented layer of the eye formed, could this be identified histologically as forebrain. The others, however, almost undoubtedly represented forebrain material, since no level behind forebrain could reasonably be expected. Hunt (1931*a*) found nervous tissue in only one of fifteen grafts of this same type, and he concluded that node tissue was probably present in the implant. This possibility is perhaps not entirely ruled out for the cases of the present study if the anterior extent of the node is considered as indefinite in morphological indication. Nevertheless, the presence of brain in these grafts from material anterior to the visible node, as well as its presence in six grafts which included lateral as well as anterior medullary plate material, suggests that the material destined to give rise to forebrain may be determined to the extent that it will self-differentiate into typical nervous tissue even in the definitive primitive-streak stage. The fact that inclusion of some node cells in grafts from the anterior level resulted, according to Hunt, in differentiation of forebrain in all cases may be due to the inclusion of more prospective forebrain material or of part of a center of rapid proliferation rather than to any influence of future notochordal material included. Since notochord never differentiated in the grafts, it is possible, although not proved, that no prospective notochord material was present in the implants.

Wetzel (1929) was able to measure the limits of the "Scheibe," future medullary plate material, as an area of thickened ectoderm, by the histological study of sections of total blastoderms. He did this for the definitive primitive-streak stage and also for earlier stages having a somewhat elongated, as opposed to a broad, primitive-streak, but with the pellucid area round rather than pear-shaped. The medullary plate material therefore becomes visibly determined before the appearance of the head-process. Since actual determination of a structure, as proved by self-differentiation, usually occurs before morphological differentiation, this would indicate that medullary plate, in part at least, may be already determined in the somewhat elongated primitive-streak stage.

Various homologies can be found between the results obtained here and those gathered concerning the determination of medullary plate in Amphibia by various German investigators. Bautzmann (1926) and others have demonstrated the power of the dorsal lip of the blastopore, future chorda-mesoderm, for the induction of the medullary plate in various species of Anura and Urodeles. Rauber (1876) first spoke of the primitive-streak of the chick as a slitlike blastopore. Since that time the homology of Hensen's node in the chick to the dorsal lip of the blastopore of Amphibia has been stressed by Adelmann (1922), Wetzel (1929), and others. In the Amphibia it is the chordamesoderm of the archenteron roof which is responsible for the medullary plate induction, rather than the endoderm. As pointed out by Gräper (1929), the process of gastrulation in the chick differs from that of Amphibia in being divided into two periods. A fertile unincubated chick egg is usually two-layered; it has both ectoderm and endoderm; gastrulation has already begun. The material which will give the future mesoderm of the embryo, however, is still on the surface and only later, with the narrowing of the broad primitive streak, comes to lie beneath the ectoderm of the future medullary plate. This it does, as shown by Wetzel (1929) by the vital staining method and by Gräper (1929) in moving-pictures of the vitally-stained blastoderm, by streaming medianly, turning under in the primitive groove region and spreading out under the ectoderm from the primitive folds. At the anterior end of the primitive-streak, at the future Hensen's node, prechordal mesoderm appears to form as a direct outgrowth from the thick knot where ectoderm and endoderm are fused. The time of origin of this mesoderm, according to Wetzel (1929), is when the broad primitive-streak is transforming into the narrow, somewhat elongated, streak. It is possible that determination of the forebrain material in the chick may occur or begin at the time of origin of the prechordal mesoderm.

There are evidences in the present study, however, which support the idea that determination of the forebrain material occurs later in development than the time of origin of the mesoderm. In grafts of the level anterior to the node in the definitive primitive-streak stage, as compared with those anterior to the head-process in the latter stage, there is a very decided decrease in the percentage which contain brain tissue. The difference is striking even if all possible cases of nervous tissue differentiation from the former stage are considered.

Also, as has been pointed out in the descriptive part of this paper, the presence of easily recognizable nervous tissue from implants of the definitive primitive-streak stage is almost entirely confined to those of a range of length of primitive-streak corresponding to that during which the head-process begins. These facts would seem to be an indication of the definite time at which the medullary plate—at least the future forebrain material—becomes determined. However, the mesoderm is already present underneath the ectoderm at this stage, and the analogies mentioned above make it not unlikely that the initial period of determination would be that of the spreading-out of the mesoderm. Also, there were a few exceptions in the author's grafts in which nervous tissue differentiated from donors with primitive-streaks shorter than the range of length at which the head-process begins. Rudnick (1932), in this laboratory, has grafts containing brain from implants lateral to node and streak in similar early stages. Forebrain has been identified in two of these by the presence of eye or epiphysis. However, all but three of Rudnick's implants from early stages were cut closer to the node and streak than mine. Also, the one graft of mine showing definitely recognizable brain material came from an implant cut very close to the streak.

The conception of a gradual process of determination culminating in the stage of the formation of the head-process would seem to fit all the data of the present study. It is conceivable that the greatly increased frequency of forebrain differentiation in grafts of implants anterior to the head-process over that in those of implants anterior to the node in the primitive-streak stage may be due to an increase in the degree of determination correlated with the appearance of the head-process. Some experiments of Goerttler (1927) led him to the opinion that there was a certain degree of determination in the medullary plate of certain species of Amphibia even before gastrulation was complete. This seems to be brought out also by the experiments of Holtfreter (1929), who found that presumptive medullary plate of blastulae and early gastrulae of various Amphibia transplanted into the lymph sacs or body cavity of an adult would differentiate into vesicles with neural-tubelike structure and possessing neurofibrils.

The absence of brain in most of the implants from early donors might be explained on the assumption that determination is taking place during this entire period but that the operation of isolating a section and transferring it to the chorio-allantoic membrane may prevent its indication in many of the grafts from earlier donors of this stage. Gräper (1926) has noted that the effect of even slight exposure to harmful agencies, such a change of temperature, light, etc., may cause a short regression in development, after which the egg may recover and again proceed in a forward direction. If this is true, we should expect a much more drastic effect from removing the blastoderm from the yolk, cutting it, and placing it on the chorio-allantoic membrane, where, as shown by Henderson (1930), it does not become incorporated for some time. There is also the possibility that the amount of prospective medullary material included was in some cases too small to differentiate. This suggestion receives some support perhaps from the fact that brain was more likely to develop in implants in which material lateral, as well as anterior, to the node was included.

It is, of course, true that in all the grafts of the primitive-streak stage mesoderm was probably included, even though it did not always differentiate. This allows for the possibility of induction occurring within the graft if we assume that the mesoderm causes induction. However, it seems unlikely that induction would begin to occur later than the spreading of mesoderm. Whether or not determination of the medullary plate material has begun earlier could perhaps be discovered by grafts from implants of prospective medullary plate ectoderm anterior and lateral to the streak in the stage when the latter is a broad tongue of cells and the blastoderm is still two-layered. Grafts of various levels of prenodal stages have been made by Hoadley (1926). While these prove self-differentiation of the level implanted, they do not prove it of single structures in the grafts. Forebrain differentiation, for example, in his grafts as well as in mine, may depend on the presence of mesoderm, since by including node or streak level he included the source of such mesoderm.

It should be noted here that the importance of the node in the process of development is not questioned. The node is the center for the proliferation of the prechordal and notochordal mesoderm; and even if determination should be present before this mesoderm is proliferated, such determination may still depend upon influences radiating from the node or from prospective node tissue at an earlier stage.

The experiments reported in this paper, for the chick, are not considered equivalent to those done in Amphibia in transplanting presumptive medullary plate into the region of presumptive epidermis. For this reason it is not considered that determination, in the sense of irreversible determination, has been demonstrated by them. However, it can be stated from these and other experiments that there is evidence of a mosaic pattern in at least part of the medullary plate material in the stage of the definitive primitive-streak. This, according to the idea expressed by Gilchrist (1929, p. 551), "indicates that determination has begun" while "capacity for regulation reveals it is still in progress." Chorio-allantoic grafting does not seem to the writer a method capable of revealing capacity for regulation.

CAPACITY OF THE HYPOPHYSIS FOR DIFFERENTIATION

The capacity of the hypophysis for differentiation in chorio-allantoic grafts has been found to be very slight from implants of donors younger than eight to nine somites, and fairly constant only from donors over fifteen somites, the time at which morphological indication appears. It is true that Hunt has reported hypophysis as differentiating in seven grafts out of twenty-five of the node level of the definitive primitive-streak stage. The possibility that this may not be hypophysis in all cases seems fairly strong and has been discussed in a previous section.

The absence of hypophysis, as previously mentioned, does not seem to be correlated with the presence or absence of any other structures in the graft with the exception of brain. Hypophysis has never been found in grafts lacking brain; but, on the other hand, the presence of normal brain tissue, even in grafts from implants containing the floor of the diencephalon (or the tissue which will give rise to it), does not insure the presence of differentiated hypophysis. Yet, it is the floor of the diencephalon which gives rise to the infundibulum, and the latter is the part of the brain normally associated with the hypophysis. It is possible, as suggested previously (Stein, 1929), that the hypophysis is peculiarly dependent for its differentiation on the normal arrangement of structures and materials and that this is prevented in some way in the grafts. Kingsbury and Adelmann (1925, p. 277) state that "one of the important factors in the development of the hypophysis is the formation of a mesoderm-free area where ectoderm of the neural tube or plate and general surface ectoderm are in intimate contact. It is believed that this mesoderm-free area is in part produced by the withdrawal from the midline of prechordal mesoderm." In cases of cyclopia this withdrawal of prechordal mesoderm does not occur. Kingsbury and Adelmann conclude that this "might in some instances prevent the formation of a mesoderm-free area, and thus would account for the failure of hypophysis to form in so many cases of cyclopia." It may be that a mesoderm-free area is not produced in grafts from early donors, that therefore the skin ectoderm and nervous ectoderm fail to make contact and the hypophysis does not develop. The idea of contact suggests dependent differentiation for which the writer has previously found some experimental evidence (Stein, 1929).

However, another possibility may be considered. Hypophysis is chiefly absent from grafts of young donors, under eight somites, and where present occurs in very small amounts and only in the largest of the grafts. It is known that differentiation continues on the chorio-allantoic membrane while cell division is retarded (Hoadley, 1929). It may be that where cleavage was more retarded, in the smaller grafts, there was simply not enough material for a recognizable expression of the hypophysis. Cleavage is more retarded in small implants than in larger ones (Hoadley, 1929; Murray and Selby, 1930). This would account for the larger amount of hypophysis present in Willier and Rawles's grafts of total blastoderms of presomite stages and in Rudnick's grafts of the median area inclusive of the node of the definitive primitive-streak stage. It may be of interest to suggest here a possible cause of the inhibition of cleavage. Sorin (1928) has shown that the yolk undergoing liquefaction beneath the chick blastoderm in the first 4–5 days of incubation gives off rays capable of stimulating mitogenetic activity in plant cells. Tissues of the whole embryo in the early part of this period and of brain in the later part do not have this effect, but the blood of the embryo of 4–5 days has it. During implantation to the chorio-allantoic membrane implants are removed from yolk and do not get an adequate blood supply for some hours (Henderson, 1930), and this happens at a time when cleavage is proceeding rapidly in the normal embryo. The absence of cleavage may therefore be due to the absence during a critical period of the agents normally stimulating it.

The degree of differentiation attained by the hypophysis is similar in all the grafts, regardless of the age of the donor. In grafts from younger implants fewer cords are formed and less hypophysial tissue is present. This naturally makes for a histological arrangement different from that in the normal embryo. The cords, being fewer in number, can scarcely fail to appear less anastamosing and to have fewer spaces filled with mesenchyme and blood capillaries.

LOCATION OF PRESUMPTIVE HYPOPHYSIAL MATERIAL

A series of grafts was made of anterior portions of head-process blastoderms, including varying amounts of the head-process, with the view of locating the material which later forms the hypophysis. Fifteen of these were examined in addition to the twenty-six of implants anterior to the head-process, and in all except one the hypophysis failed to differentiate. The one exception came from an implant limited anteriorly by the opaque area and extending posteriorly to include half of the head-process of a blastoderm in which the latter measured 0.6 mm. in length.

It had previously been found experimentally (Stein, 1929) that, in the eleven-somite stage, at least a part of the hypophysial material is located in a region of the ectoderm closely attached to the ventral surface of the forebrain and just anterior to the oral plate ectoderm. This is approximately the location given by Kingsbury (1922) from morphological studies. In a nine-somite embryo the "hypophysial area" constitutes the ventral skin ectoderm just posterior to the anterior neuropore. It may be mentioned that in two cases of embryos, one of eleven somites and one of seven somites, vitally stained by the writer anterior to Hensen's node in the definitive primitive-streak stage, the anterior neuropore and the material bordering on it were found blue upon examination. In the grafts made by Rudnick (1932) of central and lateral sections of the head-process and definitive primitive-streak stage the hypophysis, when present, was always in grafts from the central implant, which included central material from the anterior limit of the pellucid area back to the posterior boundary of Hensen's node.

The foregoing data indicate that the presumptive hypophysial material is located in the mid-line anterior to the head-process or to Hensen's node in the respective stages.

SUMMARY AND CONCLUSIONS

1. This paper treats of the location and capacity for differentiation of the prospective forebrain and hypophysial ectoderm of the chick embryo. The method of study was by chorio-allantoic grafting.

2. The donors used for the study of the forebrain were of definitive primitive-streak, head-process, and early headfold stages. It was discovered that forebrain will almost invariably differentiate from anterior levels of the head-process stage excluding all head-process material. In a few cases it also differentiated from implants of the level anterior to Hensen's node in the definitive primitive-streak stage. If the levels lateral to the streak are included with the anterior level in the implant, differentiation of nervous material in the grafts is more frequent.

The results suggest that the forebrain is determined by the time the head-process has become morphologically indicated, that the time at which it becomes indicated is a critical period in the process of determination, but that a certain degree of determination may be present even in the definitive primitive-streak stage.

The experiments also indicate that material destined to give rise to forebrain lies anterior to the node in the definitive primitive-streak stage and anterior to the head-process in the latter stage.

3. Data from previous experiments in regard to the location and capacity for differentiation of the hypophysis and prospective hypophysial ectoderm have been summarized and additional data secured.

The capacity of this tissue for differentiation is extremely limited in implants of levels anterior to the node in headfold, head-process, and early somite embryos. It rises to about 55 per cent in implants from donors of eight to twelve somites. Above fifteen somites it is considerably higher: 79 per cent from donors of fifteen to thirty somites, 94 per cent from donors of 3–5 days.

The low capacity of the hypophysis for differentiation from implants of early stages is considered. The possibilities suggested to account for it are the failure of the brain and skin ectoderm to make proper contact or the lack of sufficient cell division in the grafts.

The experiments reported here, together with those previously reported by the author and those of other investigators, indicate that in the definitive primitive-streak stage the prospective hypophysial ectoderm lies in the mid-line anterior to Hensen's node.

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THE MORPHOLOGY AND PHYSIOLOGY OF THE SALAMANDER THYROID GLAND. VII. THE EFFECT OF THE THYREO ACTIVATOR ON THYROIDECTOMIZED ANIMALS

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IN 1926 we published our first experiments (Uhlenhuth and Schwartzbach, 1926) showing that from the powder of desiccated anterior lobe of the pituitary gland of cattle a substance can be extracted which activates the thyroid gland of the larvae of salamanders, when administered in the form of intraperitoneal injections. In a series of further articles we confirmed and enlarged these findings (Uhlenhuth and Schwartzbach, 1926, 1927, 1928*a*, 1928*b*, 1929; and Schwartzbach and Uhlenhuth, 1928*a*, 1928*b*, 1928*c*, 1929*a*, 1929*b*) and finally advanced the conclusion that the anterior lobe of the pituitary gland manufactures a specific substance, the "thyreo activator," a hormone controlling the activity of the thyroid gland.

At the time of our first publication a large body of experimental work had been published, indicating that the activities of the hypophysis and thyroid gland are in some way interrelated, but no conclusive evidence existed concerning the exact nature of this relationship. The most clear-cut results had been furnished by experimentation on the larvae of amphibians, published, since 1914, by Adler (1914), Smith (1916), and Allen (1916). A summary of the conclusions to which this work led is to be found in one of Smith's articles (1920) and in a more recent review written by Allen (1929). In a general way these experiments were interpreted as meaning that in the absence of the anterior lobe normal development of the thyroid gland is impossible.

At the same time a number of experiments had been published which considerably confused the issue, being contradictory to the view that the hypophysis acts as a stimulator of thyroid activity. At first Hogben (1922–23, 1927), and later on Spaul (1925), maintained that injections of anterior lobe extracts are capable of metamorphosing the larvae of axolotls even after the thyroids of these animals had been removed. Smith (1926) found that injections of anterior lobe inhibit the metamorphosis of Colorado axolotls. And Loeb and Kaplan (1924), and later on McCordock (1928), who fed Armour's anterior lobe tablets to guinea pigs, maintained that the anterior lobe contains a hormone inhibiting the activity of the thyroid gland.

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[1] The experiments reported in this article were supported by the Julius Friedenwald Fund of the University of Maryland. A large part of the work was carried out at the Marine Biological Laboratory at Woods Hole.

Soon after the publication of our experiments, evidence was published by other investigators confirming our own findings and adopting essentially the same interpretation. The first one to repeat our experimental procedure was Leo Loeb. When working in a laboratory adjoining his own laboratory in Woods Hole, in the summer of 1928, we provided him with ample opportunity to become familiar with our results, as well as with the technique of extracting the thyreo activator from the anterior lobe. He applied our technique to his work on guinea pigs and in 1929, together with Bassett (Loeb and Bassett, 1929, 1930) published his first article confirming our findings. At this time, however, he still maintained that anterior lobe contains, in addition to a thyroid activator, a substance inhibiting thyroid activity; we had been unable to confirm his findings when feeding anterior-lobe powder prepared by ourselves (Schwartzbach and Uhlenhuth, 1928*a*). Recently Loeb, together with Siebert (Loeb and Siebert, 1930), repeated his feeding experiments on guinea pigs with the same kind of an anterior-lobe preparation used by us in our work on salamanders; he admits now that such preparations do not contain a thyroid inhibitor.

Results entirely concordant with our own findings have been published by those investigators who, like Ingram (1928, 1929) and Grant (1930, 1931), obtained activation of the thyroid gland by transplanting anterior lobes into amphibians.

The present article, and one to be published soon, will be devoted to experiments which, in our opinion, offer conclusive evidence showing that anterior-lobe injections have no visible effect in the absence of the thyroid gland. That this relation prevails was shown for the first time by Smith in 1922. We have published a short article presenting evidence in favor of the same relationship in salamanders (Schwartzbach and Uhlenhuth, 1928*b*). Recently, however, Spaul (1930) has written another article in which he reports new experiments upon the effect of anterior-lobe injections on thyroidectomized axolotls and reiterates his previous claim that anterior-lobe injections will metamorphose thyroidectomized axolotls. As Spaul makes no attempt to analyze the discrepancy between his and our own results, we wish to present, more fully, to other students of endocrine physiology our own data for critical examination.

MATERIAL

The experiments to be reported were begun in the spring of 1928. Two kinds of animals were employed. Larvae of the eastern race of *Ambystoma tigrinum* were hatched in the laboratory from spawn collected in Long Island;[2] two series—CCXCIV and CCXCV—were selected from this lot, each one consisting of the offspring of one female only. In addition to these larvae, about two hundred larvae of the western race were kept in the laboratory; all of these had hatched early in May, 1928, from spawn collected in a lake near Logan, Utah. The parentage of these larvae is unknown; the series selected from them consist, therefore, of animals which may be the offspring of one or several different females. Series CCXCIII and CCXCVII are composed of the Utah larvae.

All animals were reared under the same conditions of light, temperature, and food. At first they were kept together in lots in large trays and jars; when selected for experimental purposes, they were always kept individually in the same kind of glass jars.

PREPARATION OF ANTERIOR-LOBE POWDERS

Powders of various derivation were used in these experiments. All powders used during the summer of 1928 were made by ourselves. The anterior lobes were collected,[3] in spring and early summer, at first by the authors themselves, later on by students especially trained for this work.[4] The pituitary glands were removed, not later than 40–50 minutes after the death of the animals (oxen were used for the most part), were immediately and carefully separated into anterior and posterior lobes, and carried to the laboratory and cut, with scissors, into small pieces. These were spread out on glass plates; very soon they were sufficiently dry to be either ground up in a mortar or to be mailed, in glass-stoppered bottles, to Woods Hole, where we ground them immediately after they arrived. The powder was desiccated for 6 days in ordinary desiccators over $CaCl_2$, after which period no further appreciable loss of weight could be detected. Grinding them again each of the 6 days made them into a very fine powder.

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[2] We are greatly indebted to Professor C. S. Bishop, of the University of Rochester, for putting at our disposal the spawn of *Ambystoma tigrinum* collected on Long Island.

[3] We wish to express our gratitude to the Kaufman Packing Company of Baltimore, which gave us the most generous assistance in all our work.

The powders used in Series CCXCVII are specimens No. 13, 14, 16, 17, and 22; the glands for these powders had been collected June 22, June 28, July 6, July 9, and July 16, respectively. All of them were taken from oxen except No. 22, which was taken from cows.

These powders were used up by December, 1928; from then on, Armour's commercial powder was used (sample bought in 1928). After July 9, 1929, an anterior-lobe powder was used which Dr. Frederick Fenger, of Armour and Company, prepared especially for us, by the same technique used in our own laboratory.[5]

Among our own powders we did not find one which was ineffective when tried on normal animals. Specimens No. 13, 14, and 16, in particular, which were used with the thyroidectomized larvae, had been found potent in normal larvae.

Armour's commercial powder we found effective in metamorphosing larvae and activating the thyroids, when using it in the summer of 1926. The specimen of Fenger's preparation was tested at the same time at which it was used in the thyroidectomized larvae, in adults of *Triturus torosus*, and was found very potent in activating the thyroid.

The muscle powder was prepared from beef muscle in the same manner as the anterior-lobe powder.

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[4] Part of the glands were collected by Messrs. M. Dawson, P. C. Jett, and H. S. Shelley.

[5] The authors take pleasure to acknowledge, at this occasion, their indebtedness to Dr. Frederick Fenger for the preparation of the anterior-lobe powder.

Throughout the course of the experiments the powders were extracted in the same manner. They were added to an amount of 0.5 per cent glacial acetic acid (in 30 per cent frog Ringer), brought to a boil and filtered. The filtrate was neutralized against litmus paper by adding N/10 NaOH. The quantities of powder and of glacial acetic acid were adjusted so that, after neutralization, 1 cc. of fluid contained the extract of about 125 mg. of substance.

For the intraperitoneal injection of the extract, the larvae were anesthetized in chloretone; 10 cc. of an 0.5 per cent chloretone solution was added to 40 cc. of tap water. Three to four injections were made every week. The amount of fluid injected at one time varied with the size of the animals; the smallest amount of fluid injected was 0.2 cc., and the largest amount was 0.5 cc. The quantity of substance employed for one injection varied from 50 to 125 mg. The accuracy of the dosage was not sufficient to yield quantitative results.

EXPERIMENTS

A. AUXILIARY EXPERIMENTS

In addition to the experiment undertaken especially to test the effectiveness of anterior-lobe injections after removal of the thyroid gland, three series of normal, non-thyroidectomized salamander larvae were injected, during the same summer, with the same kind of anterior-lobe extract as were the larvae of the experiment proper; the extract was made from anterior-lobe powder prepared by ourselves. As the significance of this contribution rests upon the question whether or not the extracts employed in these experiments were really potent in causing precocious metamorphosis, it is considered important to augment the number of experiments proper by a series of auxiliary experiments showing that our anterior-lobe injections produced precocious metamorphosis in every single case.

In the first of the three experiments (Series CCXCIII) larvae of the same stock, race, and habitat were employed as those used in the experiment proper. In the other two series (CCXCIV and CCXCV) larvae of the eastern race of *Ambystoma tigrinum* were used. Full data on these experiments will be found in an article to be published soon; Table I, however, summarizes the effect of anterior-lobe injections upon metamorphosis (age and body length at which metamorphosis took place).

As Table I shows, the injected larvae metamorphosed precociously in respect to size as well as age. The first signs of metamorphosis became noticeable after about five injections; but in order to produce a shedding of the complete skin, closure of the gill slits, and disappearance of the opercular pouch, from four to twelve (in one case) injections were required (see Table II).

TABLE I

EFFECT OF ANTERIOR-LOBE INJECTIONS UPON BODY LENGTH AND AGE AT WHICH METAMORPHOSIS TOOK PLACE

EXPERIMENT	CONTROLS METAMORPHOSED AT—		EXPERIMENTALS INJECTED WITH ANTERIOR LOBE METAMORPHOSED AT—	
	Body Length (Mm.)	Age (Days)	Body Length (Mm.)	Age (Days)
CCXCIII *a*	58.2	141		
CCXCIII *aa*			38.3	105
CCXCIV *a*	50.5	164		
CCXCIV *b*			40.2	140
CCXCV *a*	51.7	128		
CCXCV *b*			43.6	119
Average	53.5	144	40.7	121

In order to examine the structure of the thyroid gland, it was necessary to kill many larvae before metamorphosis had occurred. The experimental larvae killed before metamorphosis were, throughout, in a state indicating rapid approach of metamorphosis; while the controls did not show signs of metamorphosis at all or, as in a few cases, only some slight indications of the earlier stages (such as a slight exophthalmos). By the end of August, 1928, all of the experimental larvae had metamorphosed; while by the end of September, 1928 (one month later), many of the controls were still completely larval. At this time it was necessary to remove the entire experiment from Woods Hole to Baltimore; all of the control larvae died during the journey. It is certain, however, that, had they survived, many of them would not have metamorphosed before the summer of 1929, as was actually the case with other normal larvae of this lot, which did survive the journey (see Series CCXCVII *a* 6 and

CCXCVIII *a* 2, discussed below). This condition would have still further augmented the difference between the controls and experimentals.

These three experiments show that the injections of extracts made from the same kind of anterior-lobe powders as those employed in the case of the thyroidectomized larvae were effective in every individual case.

TABLE II

NUMBER OF ANTERIOR-LOBE INJECTIONS AFTER WHICH THE ANIMALS SHED THEIR SKIN FOR THE FIRST TIME

Experiment	4	5	6	7	8	9	12	34	42	44
Normal larvae:										
CCXCV *b* 2	....	....	....	+	....	....	....	....	....	....
CCXCV *b* 3	....	....	....	....	....	....	+	....	....	....
CCXCV *b* 7	....	....	+	....	....	....	....	....	....	....
CCXCIV *b* 1	+	....	....	....	....	....	....	....	....	....
CCXCIV *b* 4	....	+	....	....	....	....	....	....	....	....
CCXCIII *b* 3	....	....	+	....	....	....	....	....	....	....
CCXCIII *b* 4	....	....	....	....	+	....	....	....	....	....
CCXCIII *b* 6	....	....	+	....	....	....	....	....	....	....
CCXCIII *b* 14	....	....	+	....	....	....	....	....	....	....
CCXCVII *h* 1	....	....	+	....	....	....	....	....	....	....
CCXCVII *h* 2	....	....	+	....	....	....	....	....	....	....
Thyroidectomized larvae:										
CCXCVII *g* 1	....	....	....	....	....	–	–	–	–	–
CCXCVII *g* 2	....	....	....	....	....	–	Died			
CCXCVII *g* 4	....	....	....	....	....	–	–	–	+	....
CCXCVII *g* 6	....	....	....	....	....	–	No further injections			

+ First skin shedding.
– No sign of metamorphosis.

B. EXPERIMENT PROPER

The entire experiment consisted of three main series: a normal control series (CCXCVII *a* and CCXCVIII *a* 2), normal larvae injected with anterior-lobe extract (CCXCVII *h*), and thyroidectomized larvae injected with anterior-lobe extract (CCXCVII *g*). The species used was the western race of *Ambystoma tigrinum* (Utah axolotl).

1. *Normal control animals.*—This experiment consisted of eight larvae—CCXCVII *a* 1–7 and CCXCVIII *a* 2. Among these, two larvae (*a* 3 and *a* 4) received eight injections of an 0.5 per cent glacial

acetic acid solution (in 30 per cent frog Ringer), boiled, filtered, and neutralized with N/10 NaOH against litmus paper, to imitate the anterior-lobe injections in every respect except the presence of anterior-lobe substance. No difference between the injected and uninjected larvae was noticed. Four animals which were still in a larval state by the end of September, 1928, died during the journey from Woods Hole to Baltimore; the remaining three animals which likewise were still larval at that time survived the journey. Of the latter three animals, one (CCXCVII *a* 2) metamorphosed in the later part of October, 1928; the other two remained in a larval condition over the winter and metamorphosed in July, 1929, at an age of about 430 days (see Table III).

TABLE III

EFFECT OF ANTERIOR-LOBE INJECTIONS UPON METAMORPHOSIS

EXPERIMENT	FIRST COMPLETE SKIN SHEDDING		KILLED OR DIED BEFORE METAMORPHOSIS	
	Age (Days)	Body Length (Mm.)	Age (Days)	Body Length (Mm.)
Normal control animals:				
CCXCVII *a* 1	....	....	143*	50·3
CCXCVII *a* 2	168	53·4	....	....
CCXCVII *a* 3	....	....	143*	46·1
CCXCVII *a* 4	....	....	143*	46.0
CCXCVII *a* 5	....	....	129†	38·7
CCXCVII *a* 6	430	57·2	....	....
CCXCVII *a* 7	....	....	143*	43·3
CCXCVIII *a* 2	427	65.8	....	....
Normal animals injected with anterior lobe:				
CCXCVII *h* 1	139	46.1	....	....
CCXCVII *h* 2	139	39·4	....	....
Thyroidectomized animals injected with anterior lobe:				
CCXCVII *g* 1	....	....	450‡	69·4
CCXCVII *g* 2	....	....	143*	37·2
CCXCVII *g* 4	450	57·0	....	....
CCXCVII *g* 6	460	69·1	....	....

* Died on the way from Woods Hole to Baltimore; no signs of metamorphosis.

† Was killed, for examination of the thyroid structure, at an early larval stage.

‡ Died from chloretone anesthesia; no signs of metamorphosis.

2. *Normal animals injected with anterior lobe.*—This series consisted of only two larvae—CCXCVII *h* 1 and 2. Although every larva injected with anterior lobe had responded promptly with meta-

morphosis, it was finally considered advisable (when the thyroidectomized larvae showed no response) to inject a few larvae with exactly the same specimens of anterior lobe as were employed in the case of the thyroidectomized larvae. Therefore, *h* 1 and *h* 2 received injections of an extract prepared from specimens No. 13, 14, and 16, powders made in our own laboratory and used in injecting the thyroidectomized larvae of Series CCXCVII *g*. The results are summarized in Table III.

The injections were started September 14, 1928, at which time the larvae were 128 days old and showed no signs of metamorphosis. After four injections the animals had become quite dark, but no distinct metamorphic characters had developed. These became suddenly pronounced, in both animals, after the fifth injection. Two days after the sixth injection, *h* 1 shed its skin for the first time. Next day both animals shed a complete skin and had to be removed from the water to be placed on moist filter paper. In contradistinction to the controls, both metamorphosed before removal to Baltimore. One animal died during the journey; the other one (*h* 2) survived and was examined September 30, 1928, 16 days after the first injection. The gills had completely disappeared, even the small black ridge which persists often for some time in normal animals, as the last vestige of the gills, had been absorbed. The gill slits were fused and grown over with skin; the opercular pouch presented the usual picture of the adult gular fold. On October 4 the animal started feeding.

Evidently extracts prepared from the powders No. 13, 14, and 16 proved conspicuously potent; they were capable of metamorphosing the larvae after six injections, 11 days after the first injection.

3. *Thyroidectomized animals injected with anterior lobe.*—This series consisted of seven larvae, CCXCVII *g* 1–7. Larvae *g* 3, 5, and 7 were used for other experimental purposes.

The thyroids were removed under chloretone anesthesia with the aid of a Zeiss binocular dissecting microscope. Careful search was made for median and accessory lateral thyroids. Only in *g* 2 could a median thyroid be detected. In the other three larvae only one lateral thyroid was found on each side. In every instance the operator, who has removed the thyroids from many hundreds of larvae of this stage, was convinced that every bit of thyroid tissue had been cleaned away. Nevertheless, as will be shown presently, the animals proved to be incompletely thyroidectomized.

The volumes of the total thyroid tissue (exclusive of the very small median thyroid) were determined and the follicles counted in each case, for future purposes. They are recorded in Tables IV and V.

Injections of anterior lobe were started 1 day after the operation (at an age of 122 days), in all four animals. Anterior lobe specimens No. 14, 13, and 16 were used for the first nine injections; these had produced metamorphosis, in the controls CCXCVII *h*, after six injections. The thyroidectomized larvae showed only a blackening, but no metamorphic effects after the ninth injection (15 days after the first injection). The results are summarized in Tables II and III.

At this time (September 22, 1928) the experiment had to be interrupted; the animals were moved to Baltimore. Larva *g* 2 died during the journey. Thirty-two days later (October 24, 1928) injections were resumed, this time with specimen No. 22, a powder prepared from cows' anterior lobes. Only *g* 1 and *g* 4 were injected, while *g* 6 was left as a control. After eight injections neither *g* 1 nor *g* 4 showed any metamorphic effects. Injections were now continued with specimen No. 17 (prepared from ox pituitary). After three injections with this specimen (twenty-one injections in all) still no effects were noticed.

After only a slight interruption, injections were now continued with Armour's commercial anterior-lobe powder. After thirteen injections with this substance (thirty-four injections in all), at an age of 283 days (February 16, 1929), neither *g* 1 nor *g* 4 showed any indications of metamorphosis. The only noticeable reaction was the pigmentary response; both larvae were deeply black.

In May three animals (*g* 1, *g* 4, and *g* 8) were removed again to Woods Hole. As no further injections had been made since February 16, the animals had become light in color. A thorough examination, on June 27, showed that the three animals appeared more larval than ever before. The gills were quite large, the fins wide, and no indication of exophthalmos was found at this time. The three animals will be discussed separately now.

In *g* 1, injections were resumed on July 10, 1929 (age 427 days), this time with an anterior-lobe powder prepared especially for us by

Dr. Frederick Fenger, of Armour and Company (specimen "Fenger, 1929"). Although this powder, when tested at the same time in adult *Triturus torosus*, was found to be a powerful thyroid activator, no metamorphic changes could be produced in the thyroidectomized larva *g* 1 after ten injections. When the animal died, at an age of 450 days, from an overdose of anesthetic, he had received 44 anterior-lobe injections in all; yet he showed no indications of an impending metamorphosis.

In *g* 4, injections were resumed July 24, 1929, at an age of 441 days. The same powder was used as in *g* 1, and eight injections of this extract were administered in all. After the fifth injection the animal began to shed his skin in small pieces; from then on he shed an abundant quantity of pieces every day, but a skin-shedding in large sheets was not observed. For fear that we might lose him before an autopsy could be made, we killed the animal after the eighth injection (forty-two injections in all), at an age of 457 days. At this time his eyes were greatly bulging, the body fin was completely absorbed, and both the gills and tail fin were much reduced in size. However, the animal had attained this stage some days previous but had ceased to advance beyond it. Neither were the gill slits fused, nor had the opercular pouch disappeared. The animal was now carefully dissected and searched for thyroid remnants. A regenerated thyroid remnant was actually found on the right side. It was removed, and its volume and the number of follicles were determined. In Tables IV and V the thyroid remnant of *g* 4 is compared with the thyroid of the same animal, removed 1 year before death, as well as with the thyroids of the two controls CCXCVII *a* 6 and CCXCVIII *a* 2, both of which metamorphosed, like *g* 4, in the summer of their second year of age. It is evident that, although the regenerated thyroid remnant was larger than the thyroids removed at an early larval stage, it was only about a sixth as large, absolutely, as the thyroid of a normal larva at that size and age. Calculated in mm.^3 thyroid tissue per cm.^3 animal volume, it was only one-third the size of a normal thyroid (Table IV). Table V shows that the thyroid remnant of *g* 4 was composed of only seventeen follicles, while the left thyroid alone of the same animal, removed at an earlier stage, consisted of fifty-seven follicles. It is probable that, while the final metamorphic reaction exhibited by this animal after a total number of forty-two anterior-lobe injections was due undoubtedly to the regeneration of a thyroid from some thyroid tissue left behind at thyroidectomy, the very slow and interrupted progress of the metamorphic changes was the result of the smallness of the remnant.

TABLE IV

SIZE OF THYROID*

EXPERIMENT	TOTAL VOLUME OF FRESH THYROID TISSUE REMOVED		MM.^{3} OF TOTAL FRESH THYROID TISSUE PER CM.^{3} OF ANIMAL VOLUME	
	At Thyroidectomy (Mm.^{3})	At Death (Mm.^{3})	At Thyroidectomy (Mm.^{3})	At Death (Mm.^{3})
Controls:				
CCXCVII *a* 6		4.140		0.302
CCXCVIII *a* 2		5.700		0.465
Experimentals:				
CCXCVII *g* 1	0.146		0.07	
CCXCVII *g* 2	0.0898		0.056	
CCXCVII *g* 4	0.128	0.662	0.099	0.127
CCXCVII *g* 6	0.123	1.295	0.088	0.084

* The calculations were carried out by Mr. David Green, of New York University.

EXPLANATION OF TABLE IV

The volume of the thyroid was calculated in the following manner. The largest and the smallest optical sections parallel to the longitudinal axis of the gland and corresponding approximately to a frontal and sagittal section, respectively, were outlined, with the aid of a camera lucida, at a linear magnification of 33. From these outline drawings, the volumes were calculated with the aid of an ellipsoid formula and utilization of the area of the sections. Many thyroids were outlined, both in fresh condition and after having been removed from the maceration fluid; from these, constants were calculated by the aid of which the volume of the fresh gland could be calculated from that of the gland run through the maceration fluid. The volumes calculated thus were divided over 36,000 to find the actual volumes.

In every one of the animals recorded inTable IV, both the left and right thyroids, if present, had been measured; median thyroids, if present, were omitted from the calculations.

Larva *g* 6 had not been given any further injections after the first nine injections. On August 5 and August 10, 1929, it was carefully examined for metamorphic changes, but none had been found. On the contrary, its eyes were observed to be conspicuously small and drawn in, much like in typically neotenous larvae. Yet on August 12, 1929, at an age of 460 days, a complete skin was found in the jar. After this first skin-shedding, metamorphosis progressed at an exceedingly rapid rate. By August 13, however, it was found, that no further reduction of its tail-fin had taken place and that the tendency to leave the water had passed off. It was killed and examined next day. No further progress had been made in the reduction of the fins and in the changes of the skin. But its gills had assumed the form of nodule-like, black stubs; the gill filaments were completely absorbed; the gill-slits were fused and grown over with skin; and the opercular pouch was replaced by the adult gular fold. In accordance with the discontinuous progress of the metamorphosis of this animal were the findings regarding its thyroids. It possessed a left and right lateral remnant, together ten times as large as the original thyroid but slightly smaller than the latter per cm.^{3} animal volume. Compared with the thyroid tissue of normal controls, however, its thyroids were only a fourth as large, absolutely as well as per cm.^{3} animal volume. The number of follicles was very small, four in the left, nine in the right remnant, as against eighty-two in the left thyroid removed at an early stage.

TABLE V

NUMBER OF FOLLICLES

EXPERIMENT	AT THYROIDECTOMY		AT DEATH	
	Left	Right	Left	Right
Controls:				
CCXCVII *a* 6	...	...	97	...
CCXCVIII *a* 2	...	...	106	...
Experimentals:				
CCXCVII *g* 1	72	...	...	...
CCXCVII *g* 2	32	...	...	...
CCXCVII *g* 4	57	...	...	17
CCXCVII *g* 6	82	...	4	9

EXPLANATION OF TABLE V

The number of follicles has been determined by a technique described in a previous article (Uhlenhuth and Karns, 1928), permitting an accurate count of the follicles.

DISCUSSION

It appears from the evidence presented above that larvae of salamanders cannot be metamorphosed, in the absence of their thyroid gland, by injections of anterior-lobe extracts. In one case, forty-four injections were not sufficient to produce even the slightest indication of metamorphic changes. If sufficient time is allowed, after operation, small remnants of thyroid tissue may regenerate; in this case metamorphosis can not only be forced by anterior-lobe extracts but may take place spontaneously, if the remnant enlarges sufficiently.

Several interesting relations may be inferred from these experiments. In the first place, it is obvious that the potency of extracts prepared from ordinary desiccated anterior-lobe powders cannot be attributed to a high iodine content of the powders. Iodized proteins, when injected intraperitoneally, can be assimilated by the salamander larvae and will produce metamorphosis even in the absence of the thyroid gland. Neither extracts of our own anterior-lobe powder nor those of Armour's commercial powder nor those of the powder prepared by Dr. Fenger contain a sufficient amount of iodine to produce metamorphosis in thyroidectomized larvae. Hence, it is quite evident that the effect of these extracts is due to a specific thyroid-activating substance contained in the anterior-lobe extract. We consider this evidence a reinforcement of evidence submitted previously and pointing in the same direction (Schwartzbach and Uhlenhuth, 1928*b*). Feeding either our own powder or Armour's commercial powder to salamander larvae proved ineffective in causing metamorphosis; whereas it should have been potent if these materials contained iodized proteins in an appreciable amount. While feeding iodized proteins to salamander larvae causes metamorphosis, it has an effect on the thyroid not even remotely similar to that following injections of anterior-lobe powders.

Secondly, we wish to reaffirm that, contrary to Spaul's findings, we must conclude, that anterior-lobe injections are ineffective in the causation of metamorphosis if the thyroids are absent. It seems to us that, with this relation true, the nature of the interrelationship of the thyroid and the hypophysis is largely stripped of the gloomy and somewhat incoherent aspect given it by Spaul. Some of the elements of the anterior lobe manufacture a specific substance, the peculiar rôle of which is to set the thyroid in action. That it produces, thereby, metamorphosis in amphibians is only a specialized case confined to amphibians alone, while its thyroid-activating capacity has been retained in the endocrine mechanism of the higher vertebrates. If the hypophysis is supposed to be a controlling device of the thyroid, it can be so only if the thyroid hormone is an inhibitor of the excretion of thyroid activator from the anterior lobe. That this relation exists is strongly suggested by the enlargement of the anterior lobe following thyroid removal.

The discrepancy between our own findings and those of Spaul remains to be explained. As pointed out above, the complete removal of the thyroids from advanced larval stages of salamanders, even in the hands of an experienced operator, is difficult, if not impossible. It is therefore necessary to begin with the injections of anterior lobe before the thyroid remnants have had time to regenerate to any appreciable size. Spaul does not state exactly how much time elapsed between the operation and the first injection of his experiments, but merely mentions that injections were started "several weeks" after operation. Moreover, in a rather detailed description of his operation technique, he merely mentions the removal of one lateral thyroid on each side. In our own experience with the Mexican axolotl, limited to only several dozen of specimens, we were struck with the fact that these animals rarely are lacking a median thyroid of considerable size and quite frequently possess several pairs of lateral thyroids. For the want of any other explanation, and because of the circumstances referred to in this paragraph, we feel inclined to believe that Spaul, in his operation, left a considerable amount of thyroid tissue behind.

We are aware of the fact that our experiments were performed on a race of *Ambystoma tigrinum* somewhat different from that used by Spaul. Unfortunately, we have not been able to obtain the Mexican axolotl. In later experiments, to be published soon, we employed large Colorado axolotls, a race more closely resembling the Mexican axolotl. These experiments are, however, entirely confirmative of the results reported in the present article and will be published in a separate article.

Spaul, in referring to our interpretation of the action of the thyroid activator, states that "no examination of the pituitary was made similar to that of the thyroid . . . ." (1930, p. 74). While this criticism appears justified, we would consider it of little weight if it means that the injection of anterior-lobe activates both the hypophysis and the thyroid of the injected animals, or activates primarily the animal's own hypophysis, and the latter in turn the thyroid. We will soon, however, publish experiments meeting Spaul's criticism, and we hope to show that they confirm our previous interpretation.

SUMMARY

1. Larvae of *Ambystoma tigrinum* were injected with extracts of anterior-lobe substance.

2. Normal larvae respond promptly to this procedure, with metamorphosis. Four to twelve injections are required to produce complete metamorphosis.

3. Thyroidectomized larvae receiving up to forty-four injections respond with metamorphosis only after the thyroid remnant has regenerated.

4. In the absence of a thyroid gland, injections of anterior lobe are physiologically ineffective.

5. The effects of anterior-lobe injections upon metamorphosis are due to the activation of the thyroid gland, caused by the thyroid activator, a specific hormone contained in the anterior lobe.

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